Studies on 2,3-diphosphoglycerate metabolism in mammals(Dissertation_全文)

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STUDIES ON 2,3-DIPHOSPHOGLYCERATE METABOLISM IN MAMMALS

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ABBREVIATIONS

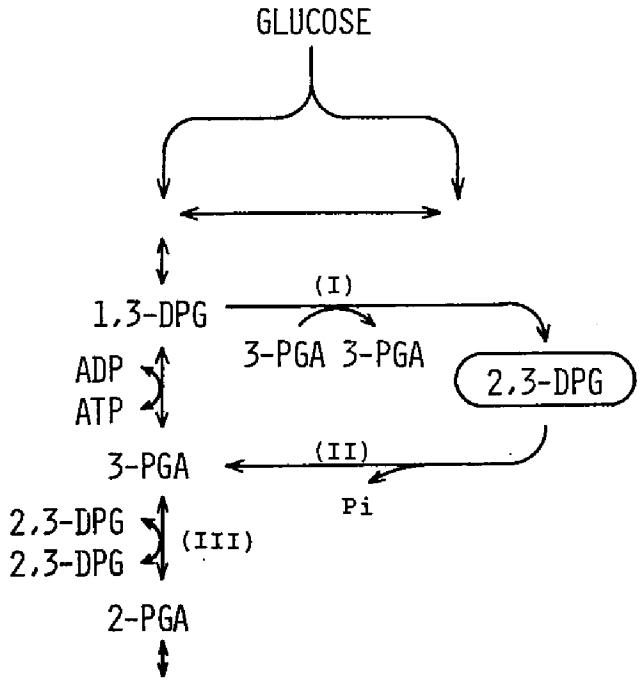
PGA mutase	Phosphoglycerate mutase (EC 2.7.5.3)
DPG mutase	Diphosphoglycerate mutase (EC 2.7.5.4)
DPG ase	2,3-Diphosphoglycerate phosphatase (EC 3.1.3.1
2,3-DPG	2,3-diphospho-D-glycerate
1,3-DPG	1,3-diphospho-D-glycerate
3-PGA	3-phospho-D-glycerate
2-PGA	2-phospho-D-glycerate
EDTA	Ethylenediaminetetraacetate
Tris	Tris(hydroxymethyl)aminomethane
DTT	Dithiothreitol
TCA	Trichloroacetic acid
TNBS	2,4,6-Trinitrobenzenesulfonate
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)

INTRODUCTION

2,3-Diphosphoglycerate(2,3-DPG) is a compound which is known as a coenzyme(or cosubstrate) of the phosphoglycerate mutase (PGA mutase: EC 2.7.5.3) reaction (Fig. 1)[1,2]. Recently, besides this role, many physiological functions of 2,3-DPG have been recognized.

In the red cells of human and most mammals, 2,3-DPG is a major component of the organic acid-soluble phosphates [3] and its concentration is much greater than would be expected of its requirement as the coenzyme of the PGA mutase reaction. In 1967, Chanutin and Curnish [4] as well as Benesch and Benesch [5] discovered that 2,3-DPG is bound to hemoglobin and thereby induces a shift to the right of the oxyhemoglobin dissociation curve. The reduction of the oxygen affinity of hemoglobin is caused by an allosteric effect of 2,3-DPG on the hemoglobin tetramer due to a preferential binding of this compound to special sites of the β-chains of the deoxyconformation [6-9]. From these findings and accumulating physiological evidences [10-13], it is now established that oxygen unloading to the tissues is decisively controlled by 2,3-DPG.

2,3-DPG not only influences the functional property of hemoglobin but also inhibits a number of red cell enzymes. Many reports have suggested that 2,3-DPG influences the rate of red cell glycolysis by its direct inhibitory effect on glycolytic enzymes: hexokinase(EC 2.7.1.1), phosphoglucomutase(EC 2.7.5.1), phosphofructokinase(EC 2.7.1.11), aldolase(EC 4.1.2.13), phos-



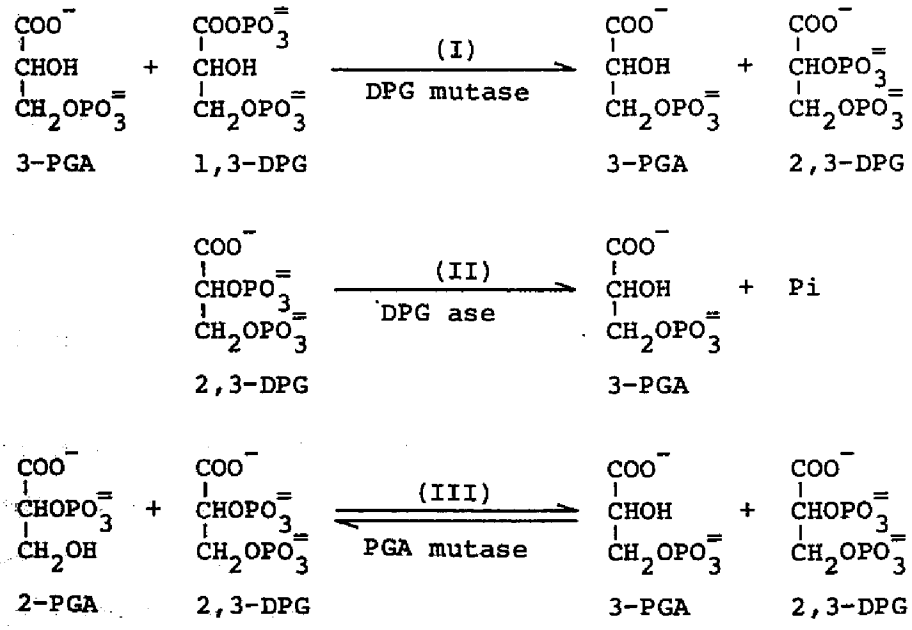


Fig. 1. Metabolism of 2,3-Diphosphoglycerate (2,3-DPG)

phoglycerate kinase(EC 2.7.2.3), diphosphoglycerate mutase(EC 2.7.5.4) and pyruvate kinase(EC 2.7.1.40) [14-19]. 2,3-DPG is also assumed to be an agent which prevents the loss of adenine nucleotides of the red cell by inhibition of adenylate deaminase(EC 3.5.4.6) [20]. Besides these enzymes, 6-phosphogluconate dehydrogenase(EC 1.1.1.43) [21], transketolase(EC 2.2.1.1) and transaldolase(EC 2.2.1.2) [22], 5-phosphoribosyl 1-pyrophosphate synthetase(EC 2.7.6.1) [23,24], AMP pyrophosphate phosphoribosyltransferase(EC 2.4.2.7) and IMP pyrophosphate phosphoribosyltransferase(EC 2.4.2.8) [24] are inhibited by 2,3-DPG.

In tissues, although the concentrations of 2,3-DPG are not so high as those of red cells (Chapter 1)[25], inhibitory effects of 2,3-DPG on several enzymes have been found. Among them are rabbit muscle pyruvate kinase [16] and phosphoglucomutase [26], beef liver phosphoglucomutase (unpublished observation) and D-glycerate dehydrogenase(EC 1.1.1.29) [27].

Such numerous studies on the physiological functions of 2,3-DPG indicate the need for regulation of the cellular level of 2,3-DPG. However, as yet there is not a good understanding of the mechanism by which the level of 2,3-DPG is controlled in animal cells. As shown in Fig. 1, the synthesis and breakdown of 2,3-DPG constitute a bypass of the phosphoglycerate kinase step of glycolysis. Rapoport has demonstrated that, in red cells, this shunt is effected by two specific enzymes, diphosphoglycerate mutase(DPG mutase: EC 2.7.5.4) and 2,3-diphosphoglycerate phosphatase (DPG ase: EC 3.1.3.13), both of which catalyze irreversible reactions (Fig. 1)[28,29]. These two enzymes are

not only present in red cells but also have been found in other tissues [30-34]. In recent years, these two enzymes have been purified from human red cells and their properties have been studied [14,35-39].

The present studies were undertaken in order to understand how the level of 2,3-DPG is regulated in animal cells. This thesis describes that DPG mutase and DPG ase activities responsible for 2,3-DPG metabolism are displayed by the same enzyme protein having PGA mutase activity, and this fact is very important as a regulatory mechanism for 2,3-DPG metabolism. In addition, the methods for the determination of 2,3-DPG in biological samples are also described.

CHAPTER 1 THE MICRODETERMINATION OF 2,3-DIPHOSPHOGLYCERATE

The method for the determination of 2,3-DPG levels in animal cells is required for better understanding of 2,3-DPG metabolism and of the physiological roles of this compound.

There are two fundamentally different enzymic procedures for assaying 2,3-DPG. The first method is based on the finding that phosphoglycolate stimulates the DPG ase activity exhibited by the PGA mutase protein [40,41]. Inorganic phosphate or 3-PGA produced is determined by the colorimetric method or by measuring the enzymically coupled oxidation of NADH. The second method utilizes the cofactor activity of 2,3-DPG in the PGA mutase reaction by the enzyme from yeast or muscle [42,43]. The product of the PGA mutase reaction has been measured spectrophotometrically at 240 nm by coupling to enolase. Although this method is the most sensitive of published procedures, only the order of the 2,3-DPG concentration in tissue extracts has been shown by its application [25,42].

In this chapter a polarimetric method to determine 2,3-DPG based upon its coenzymic activity in the PGA mutase reaction is described. The amount of 3-PGA produced from 2-PGA is measured polarimetrically as the molybdate complex [44,45]. Although this method depends on the rate of the PGA mutase reaction as in the spectrophotometric method, it does not use coupling enzymes. This should help to minimize interference. With use of this method, 2,3-DPG in hemolysates and tissue extracts was determined quantitatively.

MATERIALS AND METHODS

Enzymes and Chemicals

Crystalline yeast PGA mutase was prepared by the procedure described previously [46]. The enzyme solution (100 µg/ml) diluted by 0.5% ammonium sulfate can be stored at 5°C for three days without any loss of the activity. 2,3-DPG was obtained from Boehringer-Mannheim. Glycerol 2-phosphate was from Sigma. DL-2-PGA was synthesized by the oxidation of glycerol 2-phosphate according to the method of Kiessling [47], then it was purified on a Dowex-1Cl^- column as described by Bartlett [48].

Preparation of Extracts

Assay for 2,3-DPG was performed on the neutralized TCA supernatant of tissue extracts and hemolysates. Liver and kidney were quickly removed from a male rat and homogenized in an equal volume of ice cold water in a glass homogenizer with a Teflone pestle. The homogenates were deproteinized by the addition of 0.25 volume of 25% TCA solution and the clear supernatant was neutralized by sodium hydroxide solution. Blood withdrawn in the presence of Anglot ET as anticoagulant was washed three times with ice cold 0.9% NaCl solution. The red cells were hemolysed by the addition of an equal volume of ice cold water and freezing. After thawing, hemolysates were deproteinized and neutralized. The extracts obtained were diluted appropriately on assay.

PGA Mutase Assay

In a solution containing molybdate ions, the values of the specific rotation ([α] 1 g/ml/411.3 nm, light path = 10 cm) of

2-PGA and 3-PGA are -266 and -2780, respectively. This property can be used to analyze for the amount of 2-PGA converted to 3-PGA by PGA mutase. The reaction mixture contained 2.64 μmoles of DL-2-PGA, 200 μmoles of sodium acetate buffer(pH 5.9), 2,3-DPG sample, and 0.85 μg of the crystalline yeast PGA mutase in a final volume of 2.0 ml. The reaction was carried out at 25°C and stopped by the addition of 2 ml of 20% ammonium molybdate solution. The amount of 3-PGA produced was measured polarimetrically at 411.3 nm(Filter-3) with Union Giken High Sensitivity Polarimeter PM-70. The rate of PGA mutase reaction was not affected by the presence of the neutralaized TCA at the concentration of 1.5% in the reaction mixture. Inorganic phosphate (0.25 mM, pH 6.0) does not affect the rate.

RESULTS AND DISCUSSION

Standard Curve

Table 1 shows dependency of the PGA mutase activity on 2,3-DPG added. Activity was detected when no 2,3-DPG was added. It has been proved that this activity is not due to 2,3-DPG in the enzyme preparation but due to that in the DL-2-PGA preparation (see Chapter 2: 2,3-DPG in the DL-2-PGA preparation was found to be 0.59 mmole/mole of DL-2-PGA. This value is not required for the method in this chapter). Therefor, the initial velocity(v) at a constant DL-2-PGA concentration is expressed by Equation (I).

Table 1. Activity and 2,3-DPG Concentration

Concn of 2,3-DPG[a] (μM)	$v^b \times 10^3$	$(v - v_0^c) \times 10^3$
—	8.4	—
0.5	13.3	4.9
1.0	17.6	9.2
1.3	20.4	12.0
2.0	24.6	16.2
4.0	35.2	26.8
10.0	53.6	45.2

a The concentration in the PGA mutase reaction mixture.

b μmoles of 3-PGA formed in 1 min at 25°C.

c v_0 is the initial velocity obtained without the addition of 2,3-DPG and is 8.4×10^{-3}.

$$v = \frac{V([C_0] + [Ca])}{[C_0] + [Ca] + K} \qquad (I)$$

Here, C_0 is 2,3-DPG in the DL-2-PGA preparation and Ca is 2,3-DPG added. K is an apparent Michaelis constant of 2,3-DPG and V is the maximal velocity. Equation (I) gives Equation (II) in which v_0 is the initial velocity in the absence of 2,3-DPG added ([Ca] = 0).

$$\frac{1}{v - v_0} = \frac{[C_0] + K}{VK} \left(\frac{[C_0] + K}{[Ca]} + 1\right) \qquad (II)$$

Plot of $1/(v - v_0)$ *versus* 1/[Ca] gives a straight line. The data in Table 1 were converted to a linear line by plotting $1/(v - v_0)$ *versus* 1/[Ca] as shown in Fig. 2. This method is applicable to samples containing as little as 0.002 μmole of 2,3-DPG/ml.

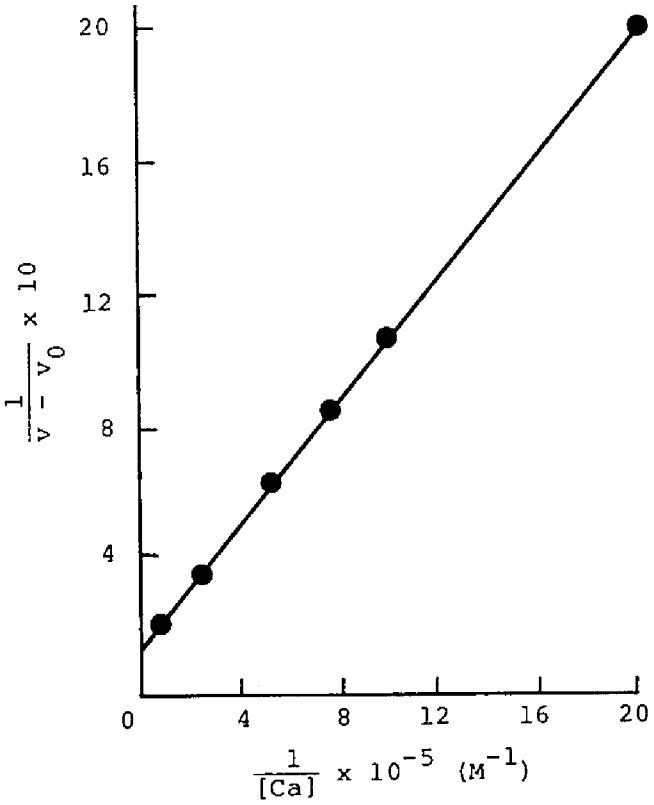


Fig. 2. Calibration line for 2,3-DPG.

The data in Table 1 were converted to linear plot. [Ca] is the concentration of 2,3-DPG in the PGA mutase reaction mixture.

Application to Extracts

The results obtained by using this method are summarized in Table 2. The amount of 2,3-DPG found was proportional to that of the extract added. Table 2 shows also the effect of the addition of known amounts of 2,3-DPG to some of the extracts and recoveries. They were found to be recovered quantitatively. These indicate that the rate of the PGA mutase reaction is not affected by substances which are introduced with the sample tested.

Table 2. 2,3-DPG Content of Tissues and Erythrocytes

Extract	Extract[a] added (ml)	2,3-DPG[b] added (μM)	2,3-DPG[b] found (μM)	Recovery (%)	2,3-DPG[c] content (av)	By the method of Rose *et al.* [40]
Rat liver	0.1	0	1.0	—	0.24	—
	0.2	0	2.0	—		
	0.1	2.0	3.2	103		
Rat kidney	0.1	0	1.6	—	0.26	—
	0.2	0	3.1	—		
Human erythrocyte	0.2	0	2.0	—	4.60	4.40
	0.6	0	5.3	—		
	0.2	2.0	3.9	95		
Rat erythrocyte	0.2	0	2.4	—	7.20	7.20
	0.6	0	7.1	—		
Goat erythrocyte	0.2	0	1.9	—	0.08	—
	0.4	0	3.7	—		
	0.2	2.0	3.9	100		
Beef erythrocyte	0.3	0	0.6	—	0.02	—
	0.6	0	1.1	—		

a Deproteinized extracts from human and rat erythrocytes were diluted appropriately.
b The final concentration in the assay system.
c μmoles of 2,3-DPG/g of wet tissue or ml of packed red cells.

The removal of TCA left in the sample was unnecessary. It was reported that the amounts of 2,3-DPG in rat liver and kidney were lower than 0.5 μmole/g of the tissue [25]. The values obtained for the erythrocytes of human and rat are in good agreement with those from many laboratories. It is a well-known fact that 2,3-DPG concentrations in the erythrocytes of ungulates, such as goat and beef, were some hundreds times lower than those of the other mammals. The method of Rose *et al.* [40] was not applicable to some of extracts and hemolysates, because of high levels of inorganic phosphate as compared with 2,3-DPG.

The DPG ase activity of yeast PGA mutase protein was found to be activated by phosphoglycolate and hydroxypyruvate phosphate [49]. To determine 2,3-DPG in human and rat erythrocytes, yeast PGA mutase and hydroxypyruvate phosphate or phosphoglycolate could be substituted for muscle PGA mutase and phosphoglycolate in the system of Rose *et al.* [40].

CHAPTER 2 2,3-DIPHOSPHOGLYCERATE AND PHOSPHOGLYCERATE MUTASE LEVELS IN ANIMAL TISSUES AND ERYTHROCYTES

It has been observed that the level of 2,3-DPG in human red cells changes in response to pathologic conditions [50-53]. Identification of the physiological significance of high 2,3-DPG contents [7] is expected to make it possible to interpret some metabolic diseases at the molecular levels [53]. This prompted the author to compare the 2,3-DPG concentration in the erythrocytes of patients with those of normal subjects. PGA mutase was chosen as a marker for glycolytic enzymes to examine whether the diseases tested are accompanied by changes in glycolytic enzyme activities or not. Polarimetric assay [54] of PGA mutase is not interfered with hemoglobin. Complete inhibition of enolase(EC 4.2.1.11) and phosphoglycerate kinase(EC 2.7.2.3) by the addition of EDTA avoids complications due to consumption of the substrate of PGA mutase(2- or 3-PGA) by these enzymes. The PGA mutase activity is not affected by EDTA. Thus, of the enzymes in erythrocytes, the PGA mutase activity is readily quantitatively determinable without any pretreatment of hemolysates. Since 2,3-DPG is also required as a coenzyme in the PGA mutase reaction, however, it is impossible to determine whether a change observed in the activity is due to a change in the amount of enzyme or in the 2,3-DPG concentration, if the hemolysate is used without dialysis. In the present work, a kinetic device made it possible to determine the 2,3-DPG concentration and the PGA mutase activity simultaneously in hemolysate

without any pretreatment. By this method, a marked increse of PGA mutase activity was observed in erythrocytes of patients with anemia and congestive heart failure.

PGA mutase is one of the enzymes functioning at the branching point of the carbohydrate metabolism and serine biosynthesis(in erythrocytes, enzymes responsible for serine biosynthesis were not observed). Three enzymes, PGA mutase, phosphoglycerate kinase and 3-PGA dehydrogenase(EC 1.1.1.95) compete for a common substrate, 3-PGA. The significance of the competition of several enzymes for a common substrate in metabolic regulation is not well known [55]. The direction and rate of 3-PGA disappearance are dependent not only on the concentration of each enzyme and on its affinity for 3-PGA but also on the concentration of the second substrate(2,3-DPG, ATP and NAD^+) of each enzyme. In view of the possible role played by this in controlling the pathways, it seems important to estimate quantitatively the factors which direct 3-PGA disappearance. The concentration of 2,3-DPG and the PGA mutase activity in crude extracts from tissues and in hemolysates were determined. The apparent Michaelis constant of 2,3-DPG was also calculated. These values are presented not only as evidence for the validity of the devised method but also as preliminary data relating to the above problem.

MATERIALS AND METHODS

Chemicals

DL-2-PGA was synthesized and purified by the methods de-

scribed in Chapter 1. The purified DL-2-PGA contained a small amount of 2,3-DPG. By means of the kinetic method described in this chapter and with the use of crystalline yeast PGA mutase, the amount of 2,3-DPG in DL-2-PGA was determined to be 0.59 mmole per mole of DL-2-PGA. 2,3-DPG and 3-PGA were purchased from Boehringer Mannheim.

PGA Mutase Assay

The reaction mixture contained 99 μmoles of DL-2-PGA, 30 μmoles of imidazole (pH 5.9), 0.3 μmole of EDTA and the amounts of 2,3-DPG indicated in the legends for the figures in a final volume of 3 ml. After the reaction was carried out at the temperatures indicated in the legends for the figures, the reaction mixture was deproteinized by the addition of 0.7 ml of 25% TCA solution and subsequent centrifugation. The supernatant(3 ml) was neutralized with 0.2 ml of 4.6 N NaOH. After 3.2 ml of 20% ammonium molybdate solution was added, the amount of 3-PGA produced was measured polarimetrically as described in Chapter 1.

Preparation of Extracts

Freshly prepared organs from male Wistar rats were chilled on ice in a beaker, washed with cold 0.9% NaCl and minced with scissors. The minced tissues were homogenized in a motor-driven Teflon homogenizer with an equal volume of 20 mM Tris buffer(pH 7.5) containing 0.1 mM DTT and 1 mM EDTA. The homogenate from rat liver was centrifuged at 100,000 x g for 60 min, and the supernatant was used for experiments, while the homogenate from rat kidney was used without centrifugation. Venous

blood was withdrawn from a cubital vein of normal subjects and patients, using heparin as an anticoagulant. Rat blood was withdrawn from the heart. Plasma and buffy coat were removed by aspiration after centrifugation of the freshly drawn heparinized blood. Red cells were washed with cold 0.9% NaCl. The packed cells were hemolysed by the addition of an equal volume of cold distilled water. The extracts (the homogenates from tissues and the hemolysates) were diluted appropriately with the above buffer to measure the PGA mutase activity and the concentration of 2,3-DPG. Aliquots of the extracts were deproteinized by the addition of 0.25 volume of 25% TCA solution and the supernatants were neutralized to measure the concentration of 2,3-DPG.

RESULTS AND DISCUSSION

Optimal pH

As shown in Fig. 3, the optimal pH of the enzyme from rat liver was pH 5.9, which was the same as those for the yeast [54] and rabbit muscle enzymes [56]. All enzymes from liver, kidney and erythrocytes of rat and human erythrocytes gave similar curves of pH dependency of the activity.

Determination of PGA Mutase Activity and Concentration of 2,3-DPG

The activity of all extracts was inhibited by 2-PGA competitively with 2,3-DPG. Because of limitations in the sensitivity of the assay method, it was impossible to vary the concentration of 2-PGA without altering the inhibitory effect. Inhibition by 2,3-DPG at high concentrations was also observed. However,

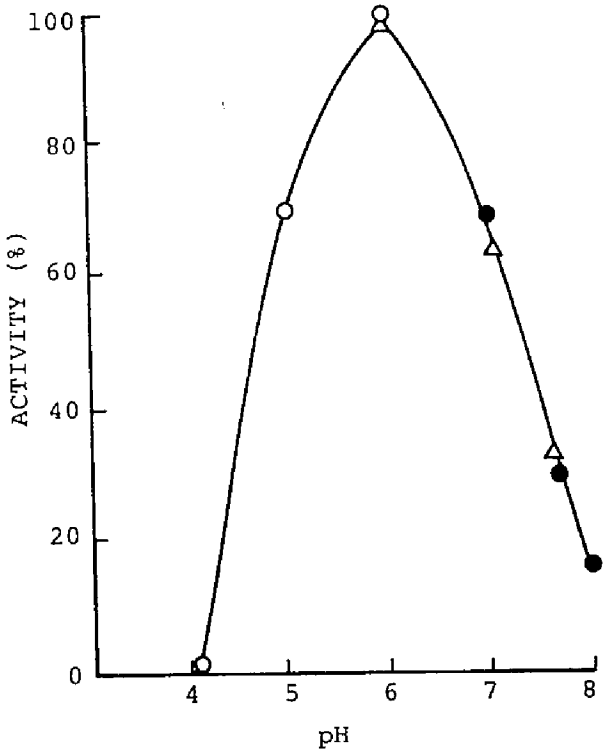


Fig. 3. Optimal pH of PGA mutase from rat liver.

The reaction mixture was as described in the text except for the presence of 0.3 μmole of 2,3-DPG. The reaction was performed at 25°C. ○, 30 μmoles of acetate buffer; △, 30 μmoles of imidazole buffer; ●, 30 μmoles of Tris buffer.

the inhibition constant for 2,3-DPG is so much larger than Km for 2,3-DPG that it was possible to vary the concentration of 2,3-DPG in the range over which the inhibition by 2,3-DPG could be neglected. Thus, the maximal activity, indicating the amount of enzyme in the extracts, can be obtained by extrapolation of the 2,3-DPG-velocity pattern to infinite concentration of 2,3-DPG. A typical pattern used to estimate the maximal activity of the extract from rat liver is shown in Fig. 4. The intersection of the two lines at a point on the 1/v axis

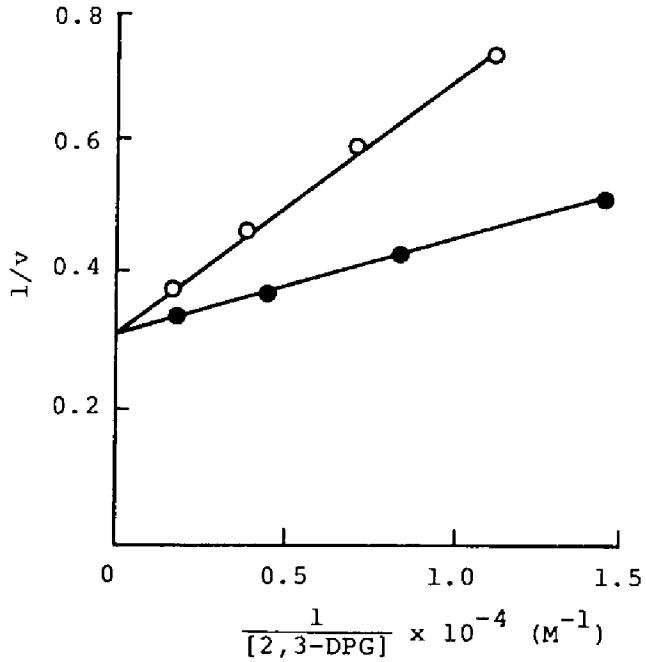


Fig. 4. Effect of 2-PGA and 2,3-DPG concentrations on PGA mutase from rat liver.

The reaction mixture was as described in the text except for the concentrations of 2-PGA and 2,3-DPG, indicated in the figure. The homogenate from rat liver prepared as described in the text was diluted with 20 mM Tris buffer(pH 7.5) containing 0.1 mM DTT and 1 mM EDTA to eliminate the effect of endogeneous 2,3-DPG. The reaction was started by adding 0.2 ml of five fold diluted homogenate and performed at 25°C. v represents μmoles of 3-PGA formed per min. ○ , [2-PGA] = 33 mM; ● , [2-PGA] = 16.5 mM.

shows that the enzyme is saturated with 2-PGA at the concentration of 2-PGA used in this chapter. Intersection at these concentrations of 2-PGA was observed for enzymes of all the extracts tested. However, dilution of homogenates from tissues or dialysis of hemolysates was required to give linear plots, since the contribution of 2,3-DPG in these extracts had to be eliminated. To avoid these pretreatments of extracts in estimating the activity of the enzyme and the concentration of 2,3-DPG, the

device of simultaneous determination of these values from a single set of experiments was performed. The purified preparation of DL-2-PGA contained a small amount of 2,3-DPG. When the activity is measured in the presence of added 2,3-DPG, which is represented by Ca, the initial velocity(v) at a constant concentration of 2-PGA is described by Equation 1.

$$v = \frac{V_{max}[C]_{total}}{[C]_{total} + Kc'} = \frac{V_{max}([C_0] + [Cx] + [Ca])}{[C_0] + [Cx] + [Ca] + Kc'} \qquad \text{Eq. 1}$$

Here, C represents 2,3-DPG. C_0 represents 2,3-DPG from the DL-2-PGA preparation and Cx is that from the extract. Kc' is the apparent Michaelis constant of 2,3-DPG (Kc' is a constant term, Kc(1 + [S]/Ksi) in which Ksi is the inhibition constant of 2-PGA [57]) and V_{max} is maximal velocity. Two mechanisms for the PGA mutase reaction pathway have been proposed [57,58]. Kinetic analysis of the mechanisms has demonstrated that the relationship between the initial velocity and the 2,3-DPG concentration obeys Equation 1 in both mechanisms under conditions at which the substrate(2-PGA) binding site of the enzyme is saturated with the substrate. For this reason Equation 1 can be used for computing data, even though the mechanisms of PGA mutases from various sources are not established. Equation 2 is derived from Equation 1 by introducing v_0, which is the initial velocity observed when 2,3-DPG is not added ([Ca] = 0).

$$\frac{1}{v - v_0} = \frac{([C_0] + [Cx] + Kc')}{V_{max}Kc'}\left(\frac{[C_0] + [Cx] + Kc'}{[Ca]} + 1\right) \qquad \text{Eq. 2}$$

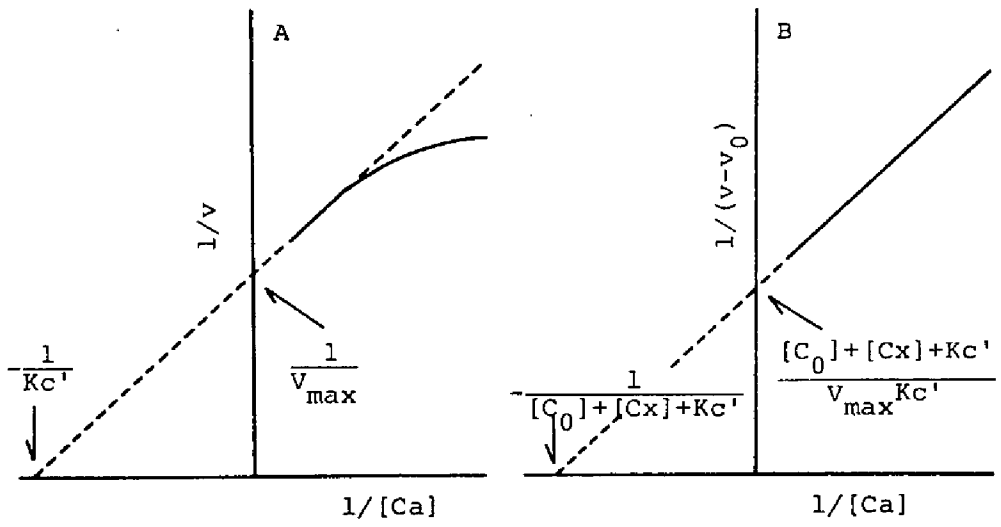


Fig. 5. Theoretical plots.

A is plotted according to Eq. 1. B is the plot according to Eq. 2.

The plot of 1/v *versus* 1/[Ca] is not linear (Fig. 5A), while the plot of $1/(v - v_0)$ *versus* 1/[Ca] (Fig. 5B) is linear at all concentrations of 2,3-DPG added. As shown in Fig. 5A, a linear plot is obtained at high concentrations of 2,3-DPG ($[Ca] \gg [C_0] + [Cx]$) and extrapolation of the linear part gives the values of V_{max} and Kc'. Using these values, the value of $([C_0] + [Cx])$ can be calculated from the intercept on the 1/[Ca] axis of Fig. 5B. The amount of 2,3-DPG in DL-2-PGA, $[C_0]$, has been determined. Thus, the concentration of 2,3-DPG, [Cx], and the activity of PGA mutase, V_{max}, in the extracts are estimated simultaneously.

Application to the Extracts

As shown in Figs. 6 and 7, the pattern of lines obtained by plotting the results for the homogenates and hemolysates coincided well with the theoretical lines in Fig. 5. The values

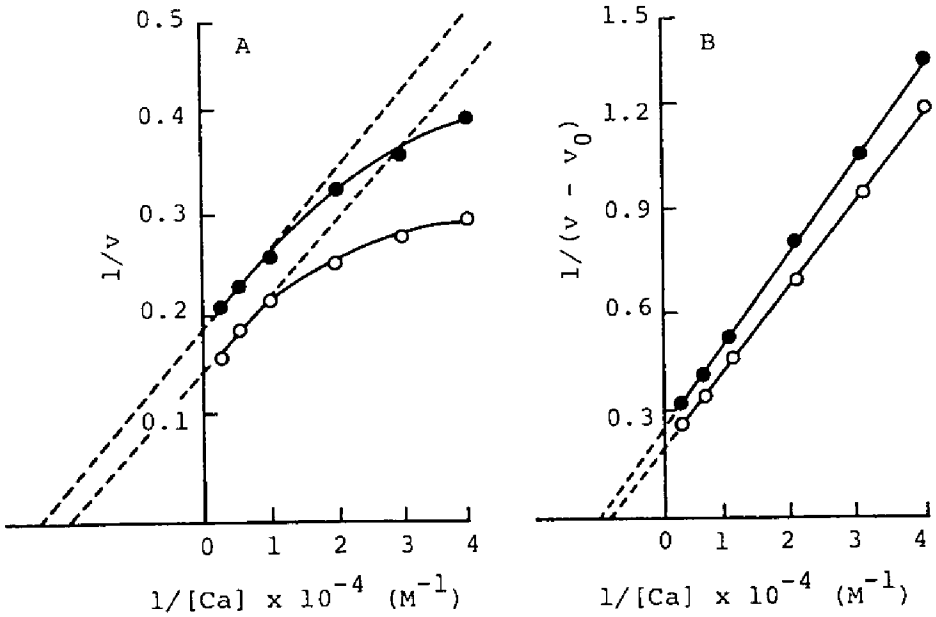


Fig. 6. Concentration of 2,3-DPG and activity of PGA mutases from rat liver and kidney.

The reaction was started by adding 0.2 ml of homogenates prepared from rat liver and rat kidney, and was performed at 10°C. v is the activity observed when 2,3-DPG was added as indicated on the abscissa. v_0 is the activity observed when 2,3-DPG was not supplemented. v and v_0 are expressed as μmoles of 3-PGA formed per min under the assay conditions. ● , homogenate from rat liver; ○ , homogenate from rat kidney.

calculated from these lines are summarized in Table 3. The concentrations of 2,3-DPG determined from its coenzymatic activity (Chapter 1) agreed well with those obtained by the method in this chapter. In the case of the homogenates from tissues, the high content of inorganic phosphate as compared with 2,3-DPG interfered with our application of the method of Rose *et al.* [40]. It was reported that the amount of 2,3-DPG in rat liver and kidney was less than 0.5 μmole per g of the tissue [42]. The concentration of 2,3-DPG found in erythrocytes is in accord with

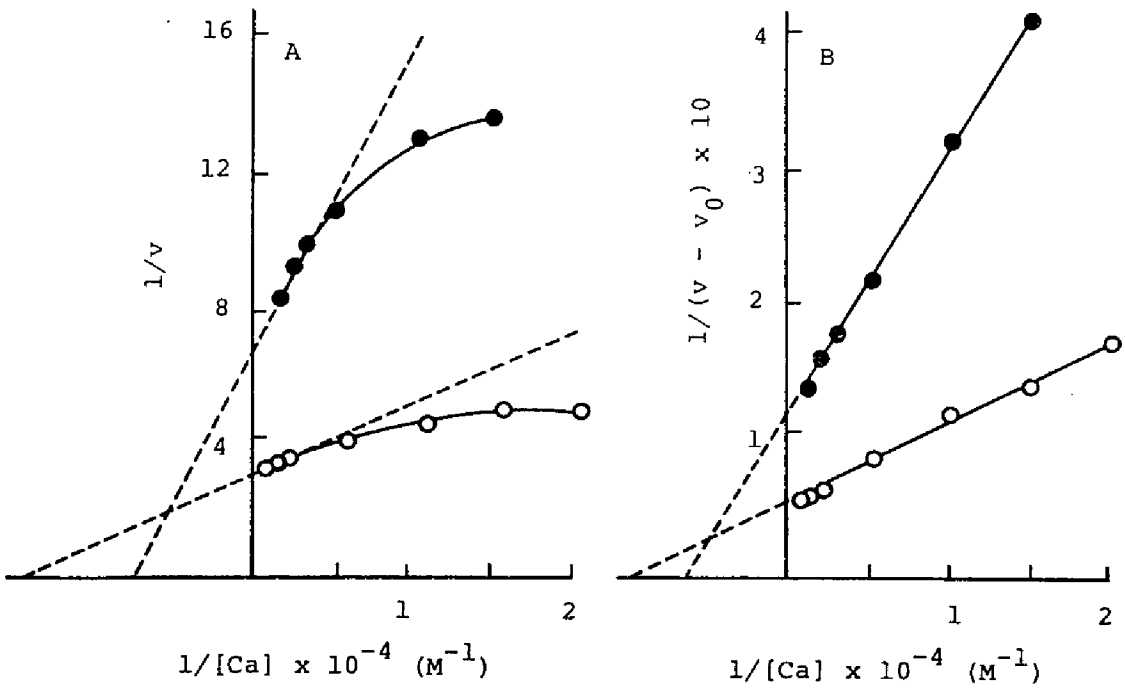


Fig. 7. Concentration of 2,3-DPG and activity of PGA mutases from human and rat erythrocytes.

The homogenate from human erythrocytes (normal subject) prepared as described in the text was diluted five-fold with 20 mM Tris buffer(pH 7.5) containing 0.1 mM DTT and 1 mM EDTA. The reaction was started by adding 0.2 ml of the diluted hemolysates and was performed at 37°C. v and v_0 are as defined in Fig. 6. ● , rat hemolysate; O , human hemolysate.

the values reported from many laboratories. The PGA mutase activity of rat erythrocytes is much lower than that of human erythrocytes. Recent studies on glycolysis in rat reticulocytes suggest that PGA mutase is a rate-limiting step because of its comparatively low concentration [59].

The results obtained by the application of this method to human blood are described in Table 4. Patients with anemia and congestive heart failure are characterized by a marked increase in the PGA mutase activity per ml of packed red cells.

Table 3. PGA mutase and Concentration of 2,3-DPG[a]

Extract	V_{max}[b]	Kc' (μM)	Concn. of 2,3-DPG[c] by the method in this paper	by coenzymic activity *	by the ** method of Rose *et al.*
Rat liver	54	68	0.25	0.24	—
Rat kidney	68	62	0.34	0.26	—
Rat erythrocyte	13	108	7.60	7.20	7.20
Human erythrocyte	32	48	4.80	4.60	4.40

a The values in this table were calculated from the plots in Figs. 6 and 7.

b Units per g of tissue or ml of packed red cells. One unit is defined as that amount of enzyme which produces 1 μmole of 3-PGA per min at the temperature indicated in Figs. 6 and 7.

c μmoles per g of tissue or ml of packed red cells.

* see Chapter 1. ** see reference [40].

This increase was observed not only in the maximal activity(V_{max}), but also in the activity(v_0), which was determined in the absence of added 2,3-DPG. It is noteworthy that the enzyme activity in whole blood of patient No. 10 was normalized, when she had markedly recovered clinically. This is not only due to the increase of hematocrit value but also to the increase in the enzyme activity of packed red cells. Although the red cells of patients No. 11 and No. 12 show high enzyme activity, the values of hematocrit are too low to maintain the activity of whole blood in the normal range. The activity per ml of whole blood in the patients with anemia was in the normal range or lower than the con-

Table 4. PGA mutase Activity and Concentration of 2,3-DPG in the Blood of Normal Subjects and Patients.

Subject			Ht.[a]	V_{max}[b]		V_0[c]		2,3-DPG[e] (mM)
No.	Age	Sex		Whole blood[d]	Red cells	Whole blood[d]	Red cells	
Group I Control subjects								
1	23	M	40	15.2	38.1	11.5	28.8	4.3
2	24	M	44	14.1	32.1	9.9	22.6	3.9
3	27	M	44	14.3	32.5	10.0	22.9	4.6
4	28	M	44	15.6	35.5	11.7	26.7	4.8
5	30	M	43	15.7	36.5	11.1	25.8	5.1
6	41	M	44	14.3	32.5	10.6	24.2	5.3
7	42	M	49	16.1	32.9	11.8	24.0	6.3
Mean±SD[f]				15.0±0.7	34.3±2.1	10.9±0.7	25.0±2.0	4.9±0.7
Group II Anemia								
8	36	F	30	14.1	46.7^{+}	10.7	35.2^{+}	4.9
9	46	F	35	14.7	42.4	11.9	34.4^{+}	4.4
10	48	F	29	10.1^{-}	34.9	7.9^{-}	27.4	8.6^{+}
			36[g]	15.7	44.0^{+}	11.4	31.9^{+}	4.6
11	27	F	17	7.7^{-}	45.2^{+}	5.9^{-}	34.9^{+}	4.8
12	47	F	14	6.1^{-}	43.0^{+}	5.3^{-}	37.0^{+}	5.3

Group III Congestive heart failure								
13	47	M	49	21.9^{+}	45.1^{+}	16.2^{+}	33.4^{+}	3.6
14	30	F	42	16.5^{+}	39.2^{+}	12.3^{+}	29.3^{+}	6.8
15	48	M	47	18.7^{+}	40.0^{+}	14.3^{+}	30.7^{+}	5.1
16	21	M	61	24.6^{+}	40.3^{+}	18.7^{+}	30.7^{+}	4.3
17	23	F	48	—	—	15.8^{+}	34.4^{+}	–
18	50	M	45	13.8	31.0	10.8	24.3	4.0
Group IV Nephritis								
19	12	M	39	13.1	33.6	10.0	25.6	6.4
20	27	M	43	14.6	34.1	10.6	24.8	4.6

a. Hematocrit. b. Units per ml of whole blood or packed cells. One unit is defined as that amount of enzyme which produces one µmole of 3-PGA per min at 37°C. c. Units per ml of whole blood or packed cells. The activity was measured without addition of 2,3-DPG. d. (V_{max} or v_0 values of red cells) x (Hematocrit/100). e. Concentration of 2,3-DPG in packed cells. f. Standard deviation. g. Clinically recovered.
+ and - represent values which have significant deviations (>10%) from the mean value of control subjects. +, higher; -, lower.

trol value, while that in the patients with congestive heart failure was higher than the control value.

Hypoxia in tissues is expected to be common in patients with both diseases. The significance of the increase in the PGA mutase activity is not yet clear,but it might be related to the increase in the physiological activity of red cell by stimulating glycolysis. It is conceivable that this serves to compensate for the decrease in the numbers of red cell of whole blood in anemia and the impaired circulation of the blood in congestive heart failure. However, analyses of glycolytic intermediates in human erythrocytes by Hamasaki *et al.* [60] have shown that PGA mutase is not involved in rate-limiting steps in glycolysis. This makes it of importance to examine whether an increase in enzyme activity occurs specifically for PGA mutase or not. Experiments to survey enzymes which may be increased in erythrocytes with these diseases are in progress.

It will be described in Chapter 3 that human red cells have three PGA mutases(peaks I, II and III) which are distinguishable by chromatography. All of these fractions also have DPG mutase(EC 2.7.5.4) and DPG ase(EC 3.1.3.13) activities. Peaks I and II have high PGA mutase activity as compared with the other two enzyme activities. Peak III is rich in DPG mutase and DPG ase activities. Determination of the fraction responsible for the increase of PGA mutase activity observed in patients remains to be done.

CHAPTER 3 Purification of Diphosphoglycerate Mutase, 2,3-Diphosphoglycerate Phosphatase and Phosphoglycerate Mutase from Human Erythrocytes
Three Enzyme Activities in One Protein

Two enzymes, diphosphoglycerate mutase(DPG mutase:EC 2.7.5.4) and 2,3-diphosphoglycerate phosphatase(DPG ase:EC 3.1.3.13), which are responsible for the 2,3-DPG metabolism in human red cells, have been purified and their enzymic properties have been investigated [14, 35-39]. The DPG mutase preparation purified by Rose [38], which showed a single protein band on electrophoresis in a polyacrylamide gel containing sodium dodecyl sulfate, had not only PGA mutase activity but also DPG ase activity in the presence of 2-phosphoglycolate which was a potent activator of the DPG ase [36]. Rosa *et al.* have shown with hemolysates from human and several animal species that DPG-mutase and DPG ase are not separable electrophoretically on a starch gel [61]. They also found a human subject who had a partial and equal deficiency of both DPG mutase and DPG ase activities in his erythrocytes [61]. Although data suggesting a resemblance in the enzymic and physical properties of DPG mutase, DPG ase and PGA mutase have been accumulated [36,38], it is not yet known whether or not the three activities are really exhibited by a common enzyme protein.

The present work was undertaken in order to purify the three enzymes from the red cells and to investigate a molecular relationship(s) among them.

MATERIALS AND METHODS

Chemicals and Enzymes

2,3-DPG, 3-PGA, DL-glyceraldehyde 3-phosphate, enolase, rabbit muscle PGA mutase and glyceraldehyde-3-phosphate dehydrogenase were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). NAD^+ was from Sigma. Hydroxylapatite was from Clarkson Chemical Co. Phosphocellulose was from Serva. Bovine serum albumin, α-chymotrypsinogen and cytochrome *c* were obtained from the Mann Research Lab. and β-lactoglobulin was from Miles Lab. DL-2-PGA was synthesized and purified by the methods described in Chapter 1. 1,3-DPG was synthesized according to the method of Rose [14]. Yeast PGA mutase was purified and crystallized as described previously [46,62].

Enzyme Assays

The activity in all instances was measured under conditions in which the initial velocities were determined.

DPG mutase, Assay I. This method was used throughout the purification. The reaction mixture contained, in a total volume of 1.0 ml: 2.5 mM DL-glyceraldehyde 3-phosphate, 40 mM potassium phosphate buffer pH 7.8, 1 mM NAD^+, 1 mM 3-PGA, 0.1 mM DTT, 2 units of glyceraldehyde-3-phosphate dehydrogenase and the enzyme. After glyceraldehyde-3-phosphate dehydrogenase was added, the mixture(0.9 ml) was incubated for 3-4 min to attain equilibrium. The 2,3-DPG synthesis was started by the addition of 0.1 ml of DPG mutase and was carried out at 37°C. The reaction was terminated by the addition of 0.25 ml

of 25% TCA. After centrifugation, the supernatant(1.0 ml) was neutralized by the addition of 0.17 ml of 1.8 M Tris. The amount of the synthesized 2,3-DPG in the neutralized supernatant was determined by using its coenzymic activity in the PGA mutase reaction as described in Chapter 1.

DPG mutase, Assay II. Isolated 1,3-DPG was used as the substrate. The reaction mixture contained, in a total volume of 1.0 ml: 0.15 mM 1,3-DPG, 0.1 mM 3-PGA, 0.1 mM DTT, 40 mM Tris-HCl buffer pH 7.5 and the enzyme. The reaction was started by the addition of purified DPG mutase and was carried out at 37 °C. After the reaction mixture was treated as in Assay I, the amount of 2,3-DPG synthesized was determined.

DPG ase, Assay I. DPG ase activity was determined by measuring the Pi liberated. The reaction mixture contained, in a total volume of 2.0 ml: 0.25 mM 2,3-DPG, 0.05 mM 2-phosphoglycolate, 0.1 mM DTT, 10 mM Tris-HCl buffer pH 7.5 and the enzyme. The reaction was started by the addition of DPG ase and was carried out at 37 °C. The reaction was terminated by the addition of 0.5 ml of 25% TCA. After centrifugation, the supernatant(2 ml) was used for determination of Pi according to the method of Bartlett [63]. With purified fractions the TCA precipitation was omitted. Assay I was used in all experiments except for the purification step with hydroxylapatite.

DPG ase, Assay II. In the purification of the enzyme with hydroxylapatite, an enolase-coupled method was utilized to measure the DPG ase. The reaction mixture contained, in a total volume of 2.5 ml: 0.5 mM 2,3-DPG, 10 mM $MgCl_2$, 0.2 mM 2-

phosphoglycolate, 40 mM Tris-HCl buffer pH 7.5, 2 µg yeast PGA mutase(component V) [62], 0.1 unit of enolase and the enzyme. The phosphatase activity was measured at 25°C by following the production of phosphoenolpyruvate (the increase in absorbance at 240 nm) with a Hitachi double-beam spectrophotometer, model 124, equipped with a recorder. Yeast PGA mutase was added to convert 3-PGA, a product of the phosphatase reaction, to 2-PGA which was led to phosphoenolpyruvate by enolase. Although yeast PGA mutase has DPG ase activity which is activated with 2-phosphoglycolate [49], the activity is a negligible quantity as compared with that of the sample used.

PGA mutase. PGA mutase activity was measured polarimetrically (see Chapter 1 and [46]). The assay mixture contained, in a total volume of 2.0 ml: 6.6 mM DL-2-PGA, 0.1 mM 2,3-DPG, 0.1 mM DTT, 20 mM Tris-HCl buffer pH 7.5 and the enzyme. The reaction was started by the addition of PGA mutase and was carried out at 37°C. The reaction was terminated by the addition of 0.47 ml of 25% TCA. After centrifugation, the supernatant (2.0 ml) was neutralized by the addition of 0.14 ml of 4.5 N NaOH. To this neutralized solution, 2.14 ml of 20% ammonium molybdate solution was added. Then the optical rotation at 411.3 nm was measured to determine the amount of 3-PGA produced, using a Union Giken High-Sensitivity Polarimeter PM-70(Filter 3). With the purified fractions TCA precipitation was omitted.

In all instances, one unit of enzyme activity is defined as the amount of enzyme which catalyzes the conversion of 1 µmole of the substrate to the product per min under the conditions

described. Specific activity is expressed as units per mg of protein.

Isoelectric Focusing

All procedures were carried out at 5°C. The column, 2.5 x 25 cm, contained a 0.7% ampholine solution with a pH range of 3 to 6, in a sucrose gradient(0-47%). DTT was added to the ampholine solution to give the concentration of 0.1 mM. The anode was the lower electrode. The pH gradient was allowed to form for 34 h in order to shorten the time during which the enzyme would be on the column. The enzyme solution was dialysed against 10 mM Tris-HCl buffer pH 7.5 containing 0.1 mM EDTA and 0.1 mM DTT (buffer A). The dialysed solution was adjusted to contain 10% sucrose and was applied to a quarter of the distance from the top of the column with a pipette extended with a piece of teflone tubing. Electrofocusing was carried out for 9 h (500 V, 1-2 mA).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the procedure of Davies [64]. Prior to electrophoresis, the enzyme was dialysed against buffer A. The dialysed solution was adjusted to contain 25% glycerol and 8% 2-mercaptoethanol, then 10 µl of the sample was layered on a gel. A current of 2 mA per gel was applied for 1.5 h at 5°C. After the gel was stained in 1% amido black 10B in 7% acetic acid for 30 min, it was washed with 7% acetic acid to destain.

Electrophoresis in a polyacrylamide gel containing sodium

dodecyl sulfate was carried out according to the procedure of Weber and Osborn [65]. The enzyme was incubated at 65°C for 30 min in the presence of 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol and 25% glycerol. After the sample was layered on a gel, a current of 6 mA per gel was applied for 4 h. The procedure for staining and destaining was as described above.

Ultracentrifugation

Ultracentrifugation was carried out in a Beckman model E analytical ultracentrifuge at 20°C. Sedimentation velocity experiments were performed with a double-sector cell. The sedimentation equilibrium experiments were performed according to the meniscus depletion method of Yphantis [66].

Protein Concentration

Protein was determined by measuring the absorbance at 280 nm. Its concentration was calculated from the absorbance at 280 nm based on the value $A^{1\%}_{280\ nm} = 10$.

RESULTS

Purification of Enzymes

All steps were carried out at 0-4°C. Fig. 8 is a flow diagram for the purification procedure. The results are summarized in Table 5.

Hemolysate. To remove the serum, 3,400 ml of the whole blood obtained from a blood bank was centrifuged at 4,500 x g for 5 min. The red cells were washed three times with an equal

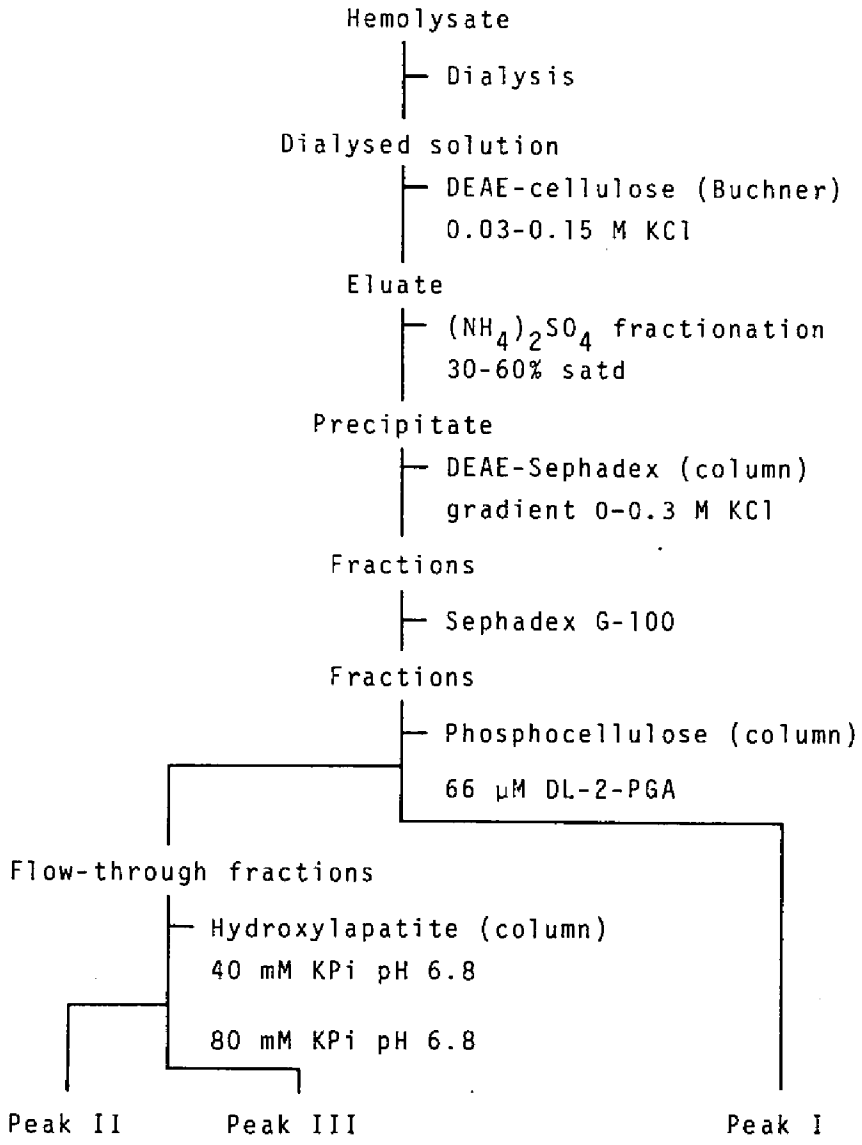


Fig. 8. Scheme for the purification of DPG mutase, DPG ase and PGA mutase from human red cells.

volume of 0.9% NaCl solution containing 0.1 mM EDTA. The buffy coat was removed with suction. The packed cells(1,450 ml) were hemolysed by adding an equal volume of 10 mM Tris-HCl buffer pH 7.5 containing 0.2 mM EDTA and 0.2 mM DTT followed by freezing

Table 5. Summary of Enzyme Purification

Fractions	Volume	Protein	Activity		
			DPG mutase	DPG ase	PGA mutase
	ml	mg	units		
Hemolysate	3,000	960,000	4,500	960	25,740
DEAE-cellulose	2,950	17,000	3,962	865	21,622
$(NH_4)_2SO_4$ fractionation	340	8,700	3,375	730	18,533
DEAE-Sephadex	610	960	3,150	663	17,504
Sephadex G-100	110	270	2,251	480	13,127
Phosphocellulose					
Flow-through	80	218	1,442	298	3,600
Peak I	60	5.4	5.4	1.5	4,120
Hydroxylapatite					
Peak II	90	37	77.2	12.5	1,780
Peak III	130	120	1,173	241	1,180

and thawing. The hemolysates were dialysed against 5 mM Tris-HCl buffer pH 7.5 containing 0.1 mM EDTA and 0.1 mM DTT (buffer B) for 24 h.

Bulk Separation on DEAE-Cellulose. To the dialysed solution, 140 g of DEAE-cellulose previously equilibrated with buffer B was added and the mixture was stirred for 3 h. The cellulose with absorbed enzymes was filtered on a Buchner funnel and washed with 3,000 ml of buffer B containing 0.03 M KCl. Then, the enzyme was eluted by washing the cellulose with 2,900 ml of buffer B containing 0.15 M KCl. The eluate was brought to 75% saturation with solid ammonium sulfate.

Specific activity			Yield		
DPG mutase	DPG ase	PGA mutase	DPG mutase	DPG ase	PGA mutase
units/mg protein			%		
0.005	0.001	0.03	100	100	100
0.233	0.051	1.27	88	90	84
0.388	0.084	2.13	75	76	72
3.28	0.69	18.23	70	69	68
8.34	1.78	48.62	50	50	51
6.61	1.37	16.51	32	31	14
1.01	0.28	763.00	0.12	0.16	16
2.09	0.34	48.11	1.7	1.3	6.9
9.78	2.01	9.83	26	25	4.6

Ammonium Sulfate Fractionation. The precipitate was collected by centrifugation at 6,900 x g for 20 min and was dissolved in 930 ml of buffer B. From the difference between the final volume(1,000 ml) of the solution and the amount of the buffer used, an estimate was made of the quantity of ammonium sulfate in the precipitate. To this solution, 190 g of solid ammonium sulfate was added to bring the salt concentration to 30% saturation. The precipitate was removed by centrifugation, and the supernatant was brought to 60% saturation by the addition of 228 g of solid ammonium sulfate.

DEAE-Sephadex Chromatography. The collected precipitate was dissolved in and dialysed against buffer B for 24 h. The

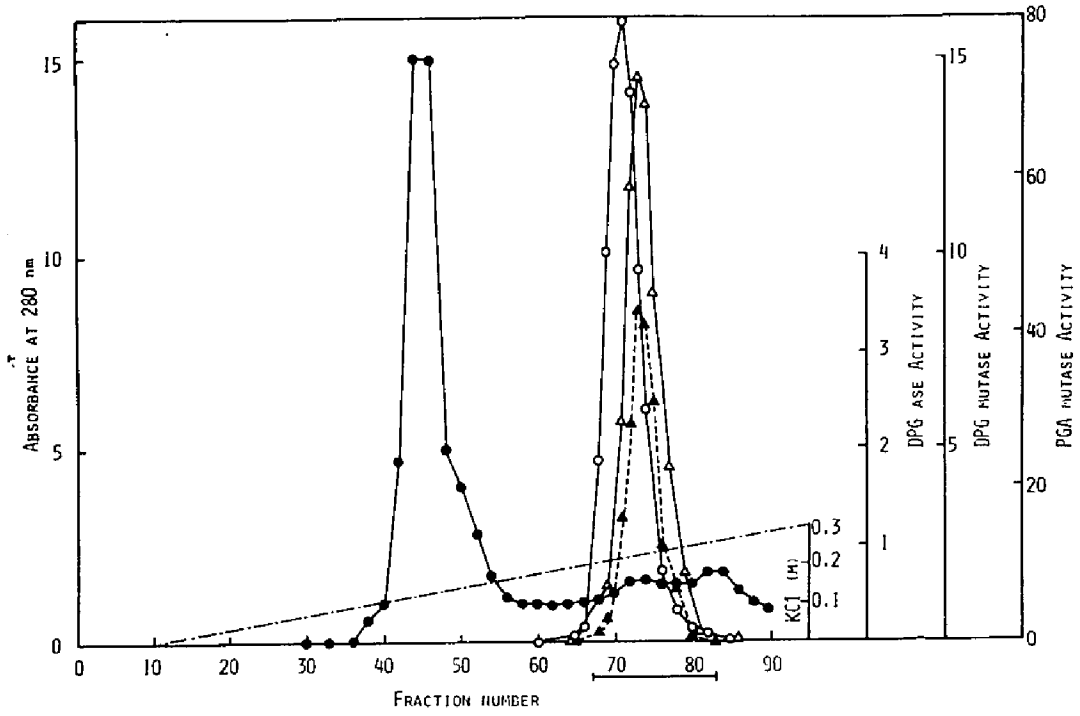


Fig. 9. Elution profile from DEAE-Sephadex.

(●—●) Absorbance at 280 nm; (△—△) DPG mutase; (▲--▲) DPG ase; (O—O) PGA mutase; (—·—·—) KCl concentration. Activities are expressed as units/ml. 4.35 g of the protein purified by ammonium sulfate fractionation was applied. Fractions were 20 ml. The flow rate was 2.8 ml/min. The horizontal line indicates the fractions pooled after elution.

dialysed solution was added to a column (4.5 x 32 cm) containing DEAE-Sephadex A-50 previously equilibrated with buffer B. The column was developed with a 4,000-ml linear gradient of KCl ranging from 0 to 0.7 M in buffer B. Fig. 9 represents a typical elution profile showing protein concentration and DPG mutase, DPG ase and PGA mutase activities. The peak for DPG mutase corresponded exactly to that for DPG ase, while the peak for PGA mutase emerged at a slightly more dilute concentration of KCl. Fractions containing the three enzyme activities were

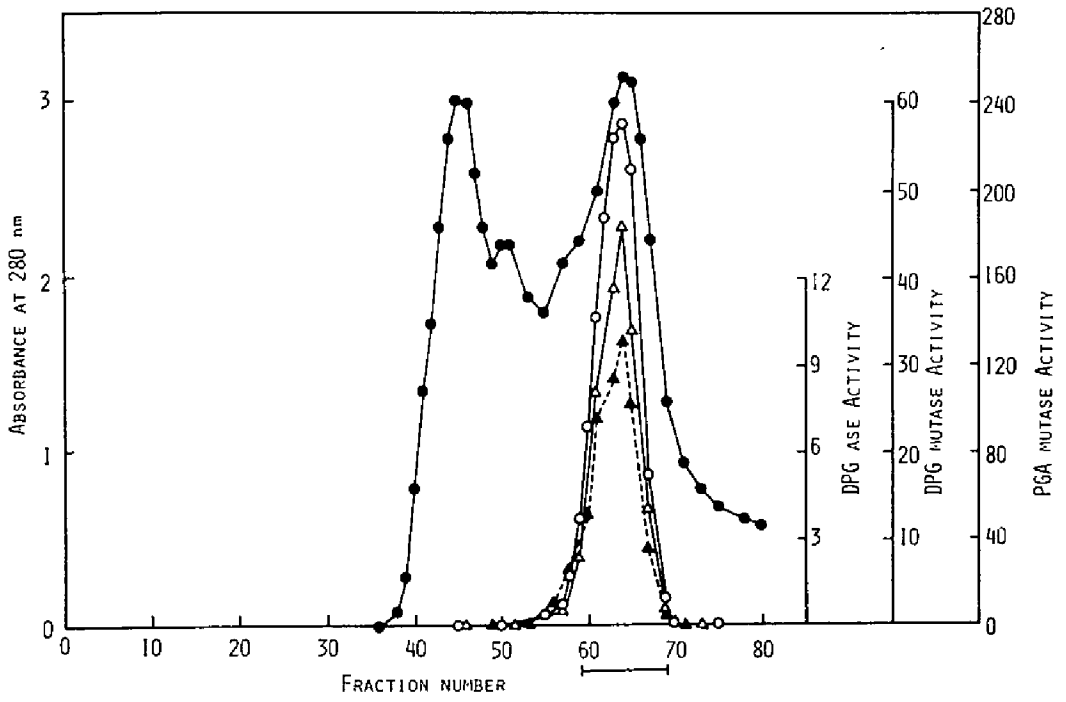


Fig. 10. Elution profile from Sephadex G-100.

(●—●) Absorbance at 280 nm; (△—△) DPG mutase; (▲--▲) DPG ase; (O—O) PGA mutase. Enzyme activities are expressed as units/ml. 480 mg of the protein purified by DEAE-Sephadex was applied. Fractions were 5 ml. The flow rate was 12 ml/h. The horizontal line indicates the fractions pooled after elution.

pooled and the protein in the pooled solution was precipitated by the addition of solid ammonium sulfate.

Sephadex G-100 Chromatography. The protein was collected by centrifugation and dissolved in and dialysed against buffer A for 1 h to complete solubilization of the protein. The dialysed solution was centrifuged at 12,000 x g for 5 min to remove insoluble materials. The clear supernatant was added to a column (2.5 x 110 cm) containing Sephadex G-100 previously equilibrated with buffer A, and the column was developed with the same buffer. As shown in Fig. 10, the three enzyme activities ap-

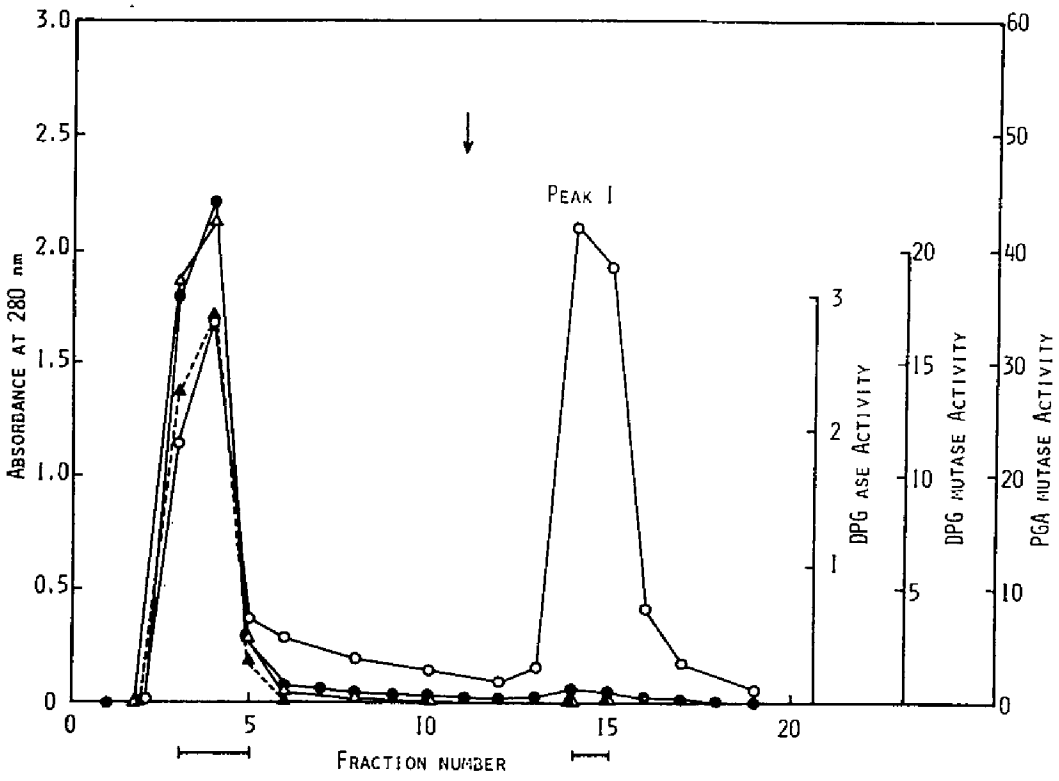


Fig. 11. Elution profile from phosphocellulose.

(●—●) Absorbance at 280 nm; (Δ—Δ) DPG mutase; (▲--▲) DPG ase; (O—O) PGA mutase. Enzyme activities are expressed as units/ml. 70 mg of the protein purified by Sephadex G-100 was applied. Fractions were 8 ml. The flow rate was 20 ml/h. At the point indicated by the arrow, the buffer containing 66 μM DL-2-PGA was used as the elution buffer. The horizontal line indicates the fractions pooled after elution. Fractions eluted with DL-2-PGA were designated as peak I.

peared in the same elution volume. The active fractions were pooled and the protein in the pooled solution was precipitated with ammonium sulfate.

Phosphocellulose Chromatography. The protein was collected by centrifugation and dissolved in and dialysed against 5 mM imidazole buffer pH 6.5 containing 0.1 mM EDTA and 0.1 mM DTT.

The buffer was added to the dialysed solution to give a final volume of 20 ml, then the whole was added to a column (1.6 x 12 cm) containing phosphocellulose previously equilibrated with the same buffer. The column was developed with the buffer containing 66 μM DL-2-PGA, a substrate of PGA mutase. Fig. 11 shows an elution profile for protein and the three enzyme activities. The bulk of DPG mutase and DPG ase activities was not absorbed by the phosphocellulose column and appeared in the flow-through fractions. These fractions contained about 45% of the total PGA mutase activity finally recovered. The PGA mutase activity absorbed on phosphocellulose was eluted with DL-2-PGA. Fractions containing the enzyme activity were pooled and the resulting solution was designated as peak I. Detectable amounts of DPG mutase and DPG ase activities were present in peak I, although these activities were very small in comparison with those in the flow-through fractions. Peak I could be eluted with a linear gradient of KCl instead of DL-2-PGA. Specific activity of peak I eluted with KCl, however, was about one-tenth of that of peak I eluted with DL-2-PGA. When the flow-through material was rechromatographed under the conditions as in Fig. 11, three activities were completely recovered in the flow-through fractions. When peak I was rechromatographed in the absence of DL-2-PGA, no detectable PGA mutase activity appeared in the flow-through fractions. The PGA mutase absorbed on the column could be eluted again with the buffer containing DL-2-PGA. In addition, the eluted fractions contained DPG mutase and DPG ase activities. The ratios of the three enzyme activities of these fractions were

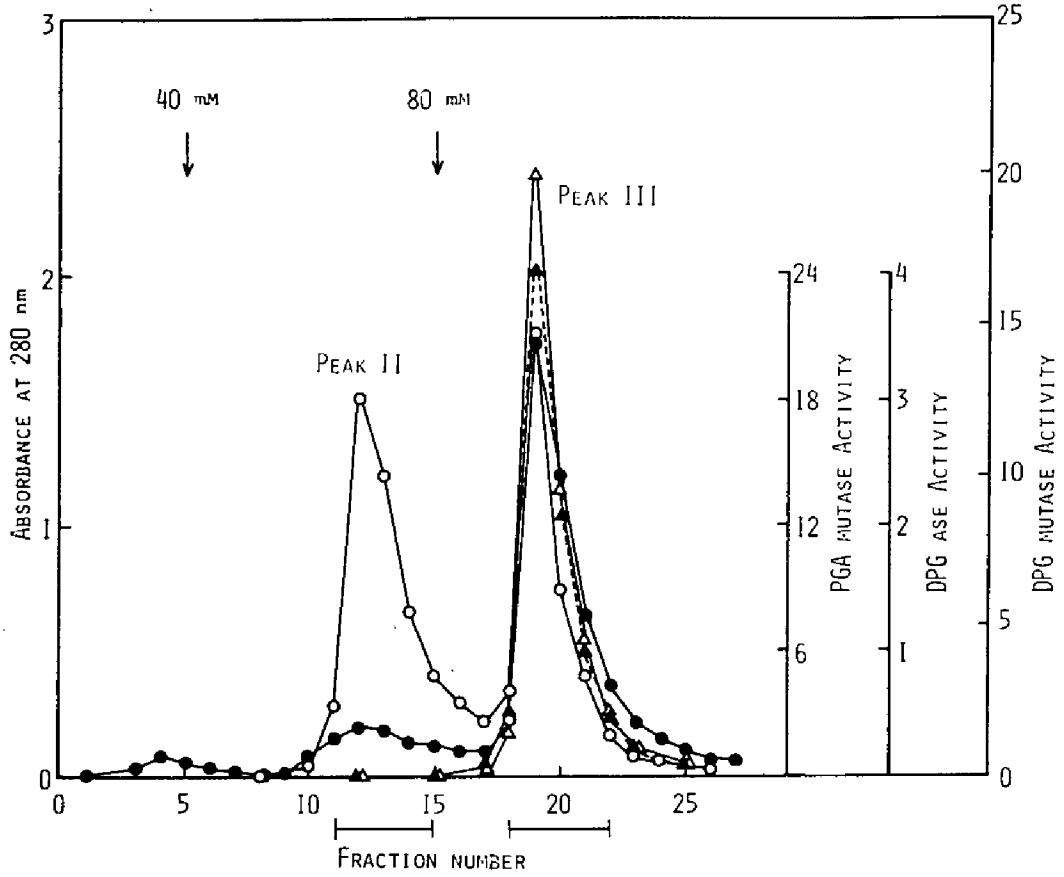


Fig. 12. Elution profile from hydroxylapatite.

(●—●) Absorbance at 280 nm; (△—△) DPG mutase; (▲---▲) DPG ase; (O—O) PGA mutase. Enzyme activities are expressed as units/ml. DPG ase was assayed with Assay II. With multiplication of the observed activity (μmole phosphoenolpyruvate produced per min) by the factor 22.8, the activity was converted to the activity of Assay I. 43 mg of the protein from the phosphocellulose flow-through fractions was applied. Fractions were 6 ml. The flow rate was 30 ml/h. The concentration of potassium phosphate buffer was elevated at the points indicated by the arrows. The horizontal line indicates the fractions pooled after elution. Active fractions eluted with 40 mM potassium phosphate buffer were designated as peak II and the active fractions eluted with 80 mM potassium phosphate buffer were designated as peak III.

nearly the same as peak I had originally. It is evident from these results that peak I is not an artificial product

in the phosphocellulose step and that the PGA mutase protein in peak I intrinsically contains the other two activities.

Hydroxylapatite Chromatography of the Flow-Through Material. The protein in the flow-through fractions was precipitated by solid ammonium sulfate and collected by centrifugation. The precipitate was dissolved in and dialysed against 10 mM potassium phosphate buffer pH 6.8 containing 0.1 mM EDTA, 0.5 mM DTT and 20% glycerol. The dialysed solution was added to a column (1.5 x 11 cm) containing hydroxylapatite previously equilibrated with the buffer. The column was washed with the same buffer, followed by stepwise elutions with 40 mM and 80 mM potassium phosphate. The elution profile of the protein and the three enzyme activities is shown in Fig. 12. Peak II, eluted with 40 mM potassium phosphate, contained 60% of the total PGA mutase activity finally recovered, while 40% of the activity appeared in peak III eluted with 80 mM potassium phosphate. Although the main DPG mutase and DPG ase activities (93-95% of the activity) appeared in peak III, these two activities were detectable also in peak II.

Isoelectric Focusing of Peak III

Peak III was subjected to isoelectric focusing for further purification, because this peak contained the main DPG mutase and DPG ase activities which probably function in the 2,3-DPG metabolism of red cells. As shown in Fig. 13, three enzyme activities were found in fractions of pH 5.0 to 5.2. These active fractions were pooled and the pooled solution was passed through Sephadex G-50 column, equilibrated with the buffer A, to remove ampholine and sucrose. The recovery of the three activities

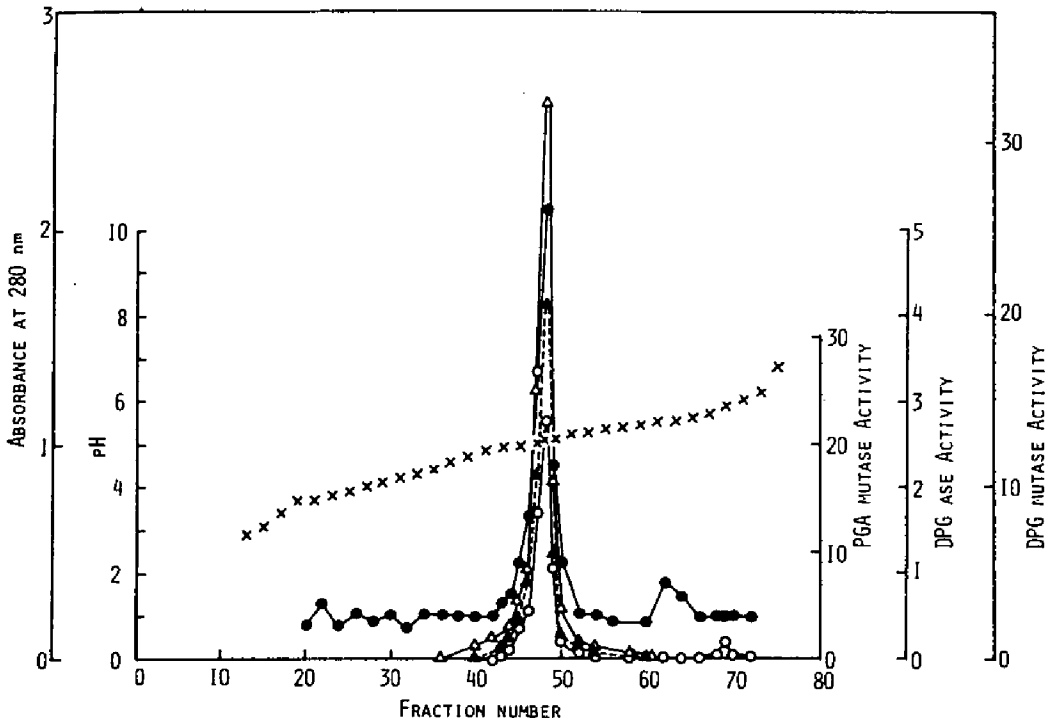


Fig. 13. Isoelectric focusing pattern of peak III.

(●—●) Absorbance at 280 nm; (△—△) DPG mutase; (▲---▲) DPG ase; (O—O) PGA mutase; (X) pH of the fractions at 25°C. Enzyme activities are expressed as units/ml. 11.5 mg of the protein of peak III purified by hydroxylapatite was applied. Fractions were 1.5 ml.

was 60% and A_{280} : A_{260} of the final preparation was 1.81. Little difference between the final preparation and peak III was found in the ratios of the three enzyme activities and in the specific activities. In fractions around pH 6.0, 3% of the total PGA mutase activity was found. This activity is probably due to the contamination of peak II and it corresponds to the PGA mutase described by the other investigators [37,39]. The DPG mutase and DPG ase activities in these fractions were not measured because of lack of enough sample.

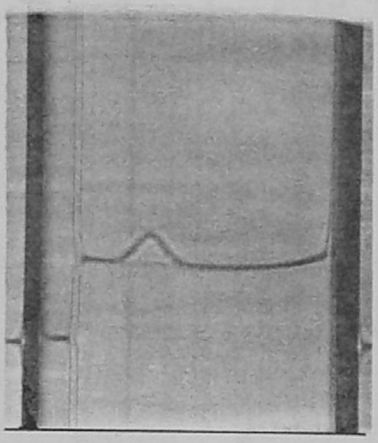


Fig. 14. Sedimentation pattern of the purified enzyme.

The enzyme obtained by the isoelectric focusing of peak III was used. The protein was 3.15 mg/ml in buffer A containing 0.1 M KCl. Migration is from left to right. This photograph was taken 50 min after reaching a speed of 60,000 rpm. The schlieren plate angle was 55°.

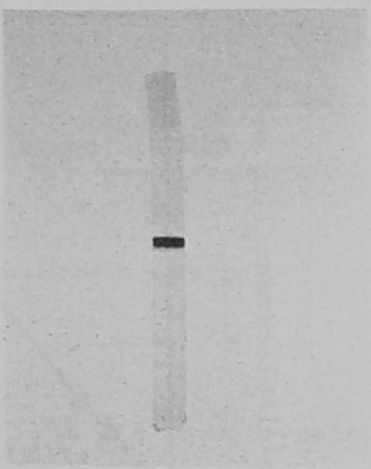


Fig. 15. Electrophoretic pattern of the purified enzyme in polyacrylamide gel.

The enzyme obtained by the isoelectric focusing of peak III was used. The protein applied was 13 µg. Migration is from top to bottom.

Criteria of Purity

The enzyme preparation obtained by the isoelectric focusing of peak III was analysed for purity using an ultracentrifuge and polyacrylamide gel electrophoresis. Fig. 14 shows a typical sedimentation pattern which gives a single and symmetrical boundary with a $s_{20,w}$ value of 4.1 S.

The enzyme preparation gave a sharp single band on polyacrylamide gel electrophoresis (Fig. 15). It was also homogeneous on electrophoresis in a polyacrylamide gel containing sodium dodecyl sulfate (see Fig. 17B).

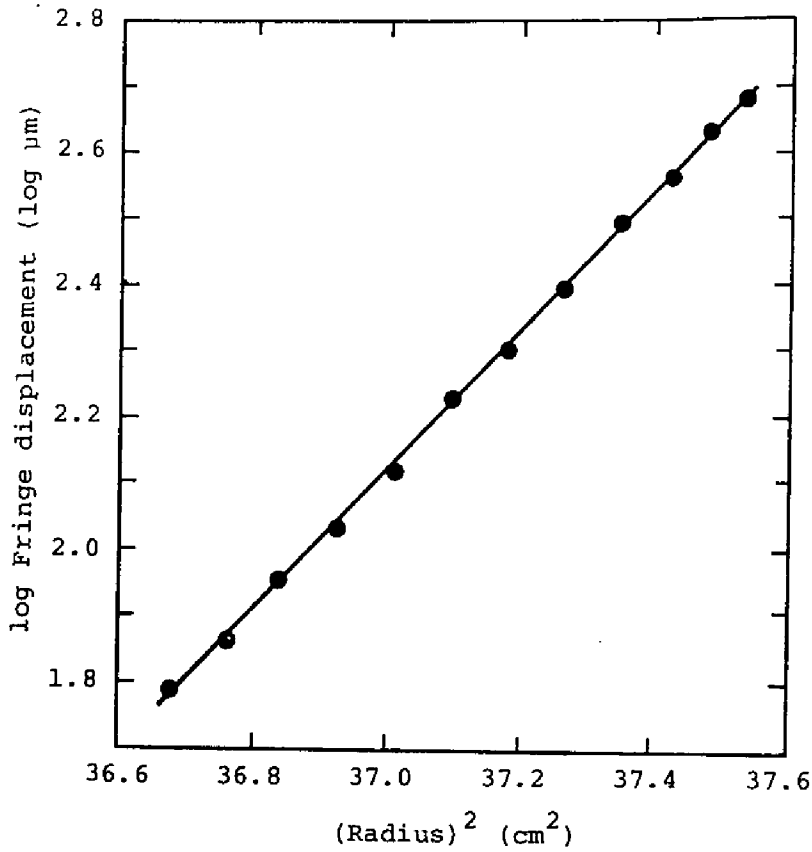


Fig. 16. Analytical graph of the sedimentation equilibrium pattern.

The enzyme obtained by the isoelectric focusing of peak III was used. The protein was 300 μg/ml in buffer A containing 0.1 M KCl. The logarithm of the fringe displacement is plotted against the square of the radial position. Data were obtained from a picture taken with a Rayleigh interference optical system 9 h after reaching a speed of 26,000 rpm.

Molecular Weight and Subunit

The molecular weight of peaks I, II and III was determined by gel filtration on Sephadex G-100 under the same conditions as in Fig. 10. A standard curve to estimate the molecular weight of samples was prepared using proteins of known molecular weights: β-lactoglobulin(35,000); ovalbumin(47,000); rabbit muscle PGA mu-

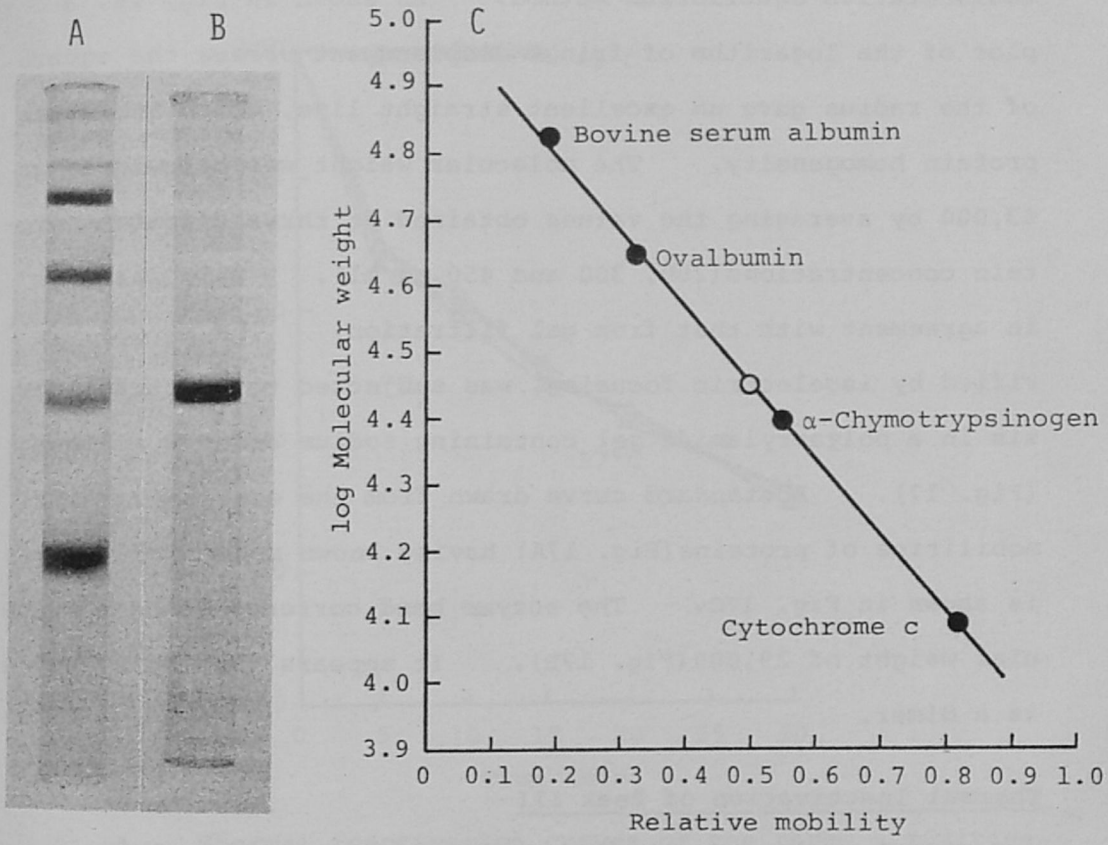


Fig. 17. Electrophoretic pattern of the purified enzyme in polyacrylamide gel electrophoresis containing sodium dodecyl sulfate.

(A) Standards (top to bottom) contain bovine serum albumin (67,000), ovalbumin(47,000), α-chymotrypsinogen(25,000) and cytochrome c(12,400). (B) Enzyme obtained by the isoelectric focusing of peak III (5 μg). (C) Standard curve drawn from the mobilities of standard proteins. Migration is from top to bottom. (●) Standard proteins; (O) sample protein.

tase(57,000); bovine serum albumin(67,000); yeast PGA mutase(110,700). The three activities of each peak were found in the same elution volume which corresponded to a molecular weight 60,000.

The molecular weight of the homogeneous preparation obtained by the isoelectric focusing of peak III was determined using the

sedimentation equilibrium method. As shown in Fig. 16, a plot of the logarithm of fringe displacement *versus* the square of the radius gave an excellent straight line, which indicates protein homogeneity. The molecular weight was calculated as 63,000 by averaging the values obtained at three different protein concentrations(200, 300 and 450 μg/ml). This value is in agreement with that from gel filtration. The enzyme, purified by isoelectric focusing, was subjected to electrophoresis in a polyacrylamide gel containing sodium dodecyl sulfate (Fig. 17). A standard curve drawn from the electrophoretic mobilities of proteins(Fig. 17A) having known molecular weights is shown in Fig. 17C. The enzyme band corresponds to a molecular weight of 29,000(Fig. 17B). It appears that this enzyme is a dimer.

Thermal Inactivation of Peak III

As shown in Fig. 18, three activities of peak III were completely stable on heating the enzyme at 45°C for 15 min. When the enzyme was heated at 57°C, three activities were lost at the same rate, which indicates that they are exhibited by the same enzyme protein.

Enzymic Properties of Peak III

Several compounds have been found to have effects on DPG mutase and DPG ase [14,36,38,67]. Effects of these compounds were tested on the two activities of peak III (Table 6). Peak III had a DPG ase activity of 0.008 unit per mg of protein in the absence of an activator. This activity was activated

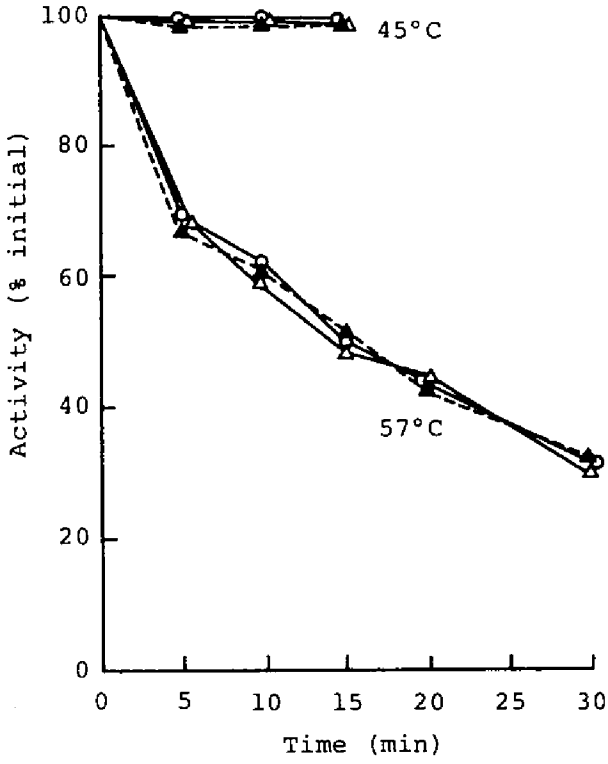


Fig. 18. Thermal inactivation curves of the three activities of peak III.

(△—△) DPG mutase; (▲--▲) DPG ase; (○—○) PGA mutase. The protein concentration of peak III was 125 μg/ml in buffer A. The sample solution(1 ml) was heated at the indicated temperatures and aliquots were removed at intervals to assay the three enzymes. DPG mutase was assayed with Assay I.

strongly by bisulfite in the presence of KCl or by 2-phosphoglycolate, while it was inhibited by 3-PGA. The DPG mutase activity was inhibited by 2,3-DPG, potassium phosphate, bisulfite or 2-phosphoglycolate. These effects are in agreement with the results reported previously [14,36,38,67], although they can not be compared quantitatively with previous data because of the different assay conditions.

Table 6. Effects of Compounds on the DPG ase and DPG mutase of Peak III.

DPG ase was assayed with Assay I using 1.1-160 μg of the enzyme(peak III) and DPG mutase was assayed with Assay II using 0.5 μg of the enzyme(peak III). The control activity of DPG ase was 0.008 μmole Pi produced per min per mg protein and that of DPG mutase was 16.6 μmole of 2,3-DPG produced per min per mg protein.

Enzyme	Compounds	Concn (mM)	Relative activity
DPG ase	Control	—	1.0
	3-PGA	0.1	0.4
	KCl + $NaHSO_3$	100 10	44
	KCl + $NaHSO_3$	100 50	86
	2-phospho-glycolate	0.02	180
	2-phospho-glycolate	0.2	810
DPG mutase	Control	—	1.0
	2,3-DPG	0.1	0.61
	KPi	0.4	0.62
	$NaHSO_3$	6.0	0.53
	2-phospho-glycolate	0.4	0.68

PGA mutase in Peaks I, II and III

Thermal Inactivation. Thermal inactivation studies were performed in order to compare properties of the PGA mutase activities of peaks I, II and III. As shown in Fig. 19A, the PGA mutase activity of peak III was quite stable at 45°C, while the

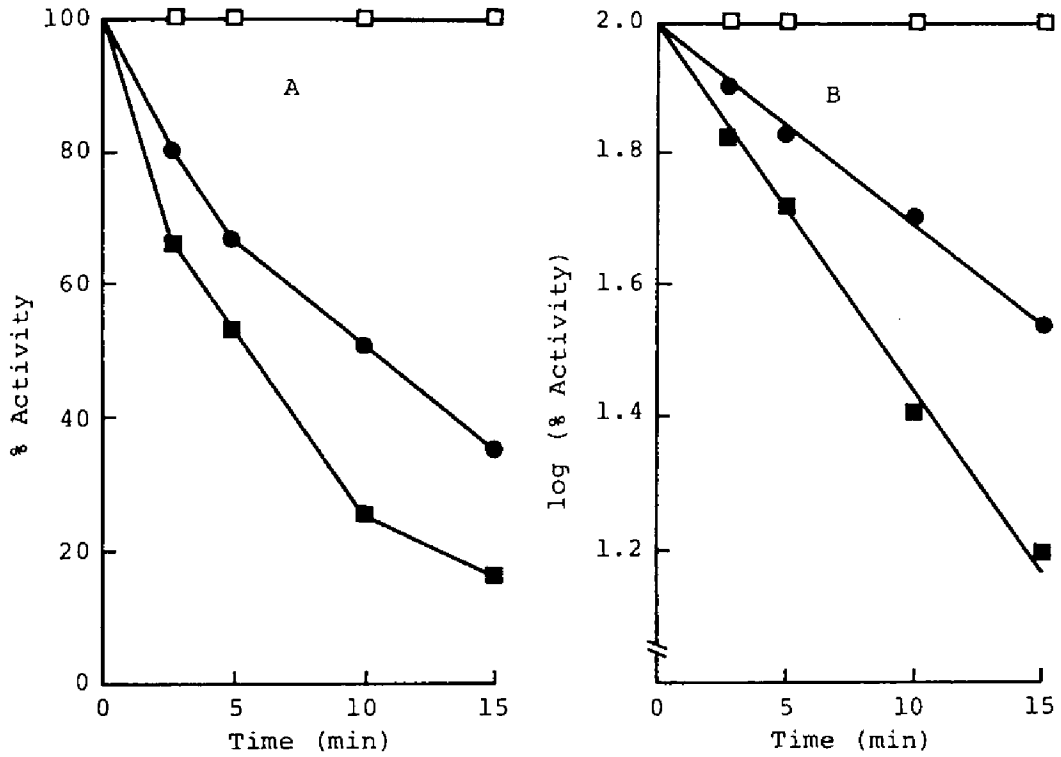


Fig. 19. Thermal inactivation curves of the PGA mutase of peaks I, II and III.

(■—■) Peak I; (●—●) peak II; (□—□) peak III. The protein concentration was 3 μg/ml in peak I, 40 μg/ml in peak II and 125 μg/ml in peak III in buffer A. Sample solutions (1 ml each) were heated at 45°C and aliquots were removed at intervals to assay the PGA mutase. The results in (A) were replotted in (B) in order to estimate the first-order rate constants for the thermal inactivation.

activity of peaks I and II was lost very rapidly. The activity of peak II, however, seems to be more stable than that of peak I. The first-order rate constant for the thermal inactivation process was calculated as 0, 0.069 and 0.126 min^{-1} for peaks III, II and I, respectively(Fig. 19B). The lack of the enough protein in peaks I and II and the marked difference in the specific activity between three peaks forced us to use the

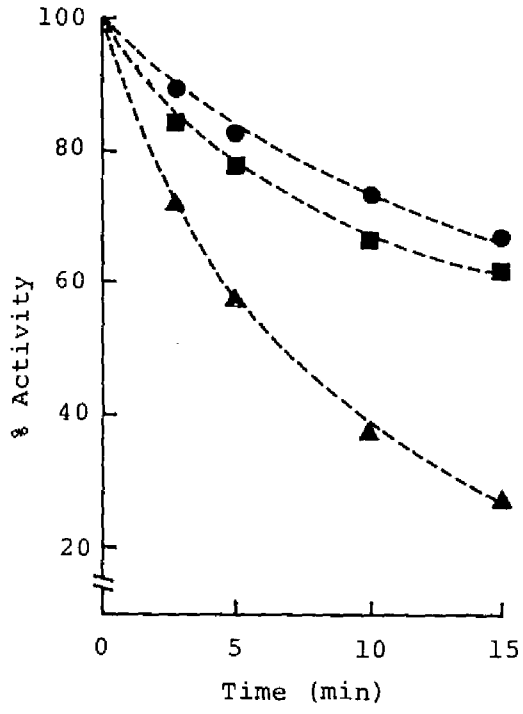


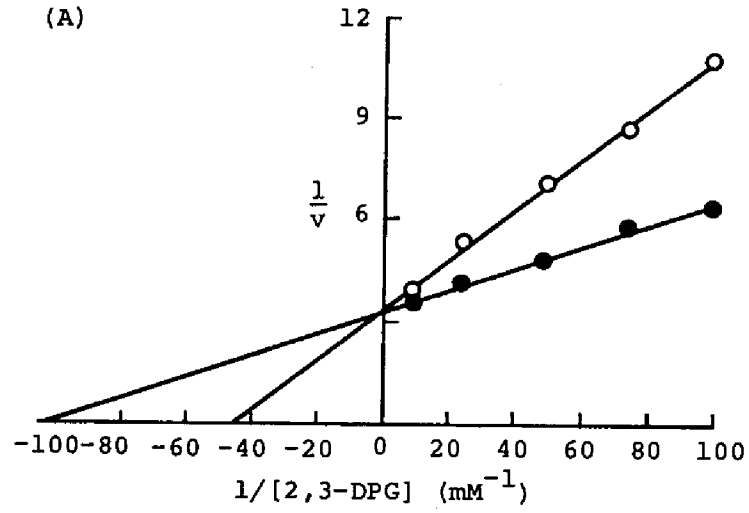
Fig. 20. Thermal inactivation curves of the preparation obtained by mixing the peaks.

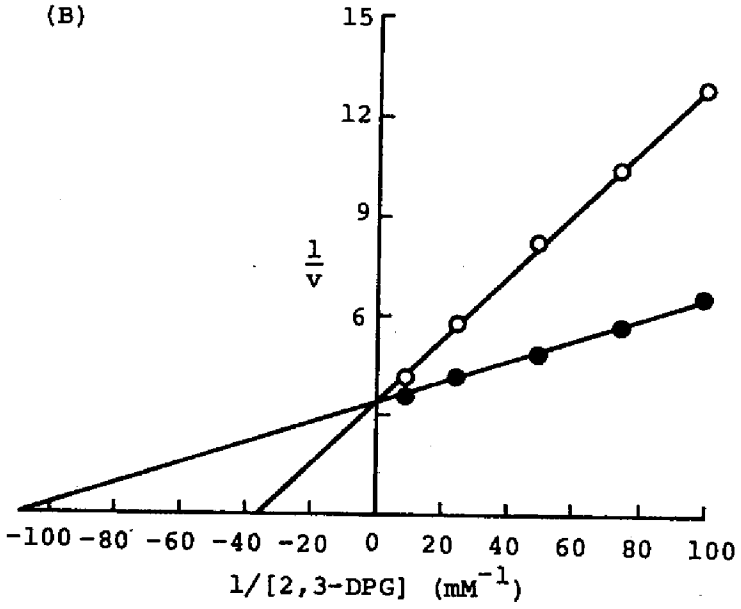
(▲ , ■ , ●) Experimental results; (-----) theoretical curves. (▲) Peaks I and II were mixed to give an enzyme solution in which 42% of the total activity was from peak I and 58% was from peak II. The protein concentration of this enzyme solution was 43 μg/ml(3 μg from peak I and 40 μg from peak II). (■) Peaks I and III were mixed to give an enzyme solution in which 46% of the total activity was from peak I and 54% was from peak III. The protein concentration of the enzyme solution was 128 μg/ml(3 μg from peak I and 125 μg from peak III). (●) Peaks II and III were mixed to give an enzyme solution in which 54% of the total activity was from peak II and 46% was from peak III. The protein concentration of the enzyme solution was 165 μg/ml(40 μg from peak II and 125 μg from peak III). The proteins were dissolved in buffer A. Enzyme solutions (1 ml each) were heated at 45°C and aliquots were removed at intervals to assay the PGA mutase. Theoretical curves were drawn as described in the text.

different protein concentration in each experiment. To exclude the effect due to the dependency of thermal stability on pro-

tein concentration, peaks I and II, I and III, or II and III were mixed in a known ratio, approximately 1 : 1 in activity. Their theoretical inactivation curves are drawn with a dotted line in Fig. 20. These curves were calculated by using the first-order rate constants in Fig. 19B based on the assumption that the PGA mutase activity of each peak in the mixed solution was lost at the same rate as when each peak alone was heated. The experimental results fitted the theoretical curves as in Fig. 20. This indicates that the protein of each peak has no effect on the thermal stability of the PGA mutase activity in the other peaks and that the activity of peak III is essentially more stable against thermal inactivation than is activity of peak I or II.

Apparent Km for 2,3-DPG. Rose has reported that the PGA mutase activity in the purified DPG mutase preparation is enhanced by the addition of a high level of 2,3-DPG, within which range the main PGA mutase in red cells is not affected [38]. To examine this with our preparations, kinetic studies on PGA mutasesof three peaks were carried out. As shown in Fig. 21, the PGA mutase activities of the three peaks suffered substrate inhibition, which was competitive with respect to 2,3-DPG. This type of inhibition has been observed with PGA mutases from other sources [57,68,69](Chapter 2). As summarized in Table 7, no significant difference in the apparent Km values was observed between peaks I and II, while the values obtained for peak III were about 3-fold larger than those of peaks I and II. This


(A)
12
9
1/v
6
-100 -80 -60 -40 -20 0 20 40 60 80 100
1/[2,3-DPG] (mM^{-1})


(B)
15
12
9
1/v
6
-100 -80 -60 -40 -20 0 20 40 60 80 100
1/[2,3-DPG] (mM^{-1})

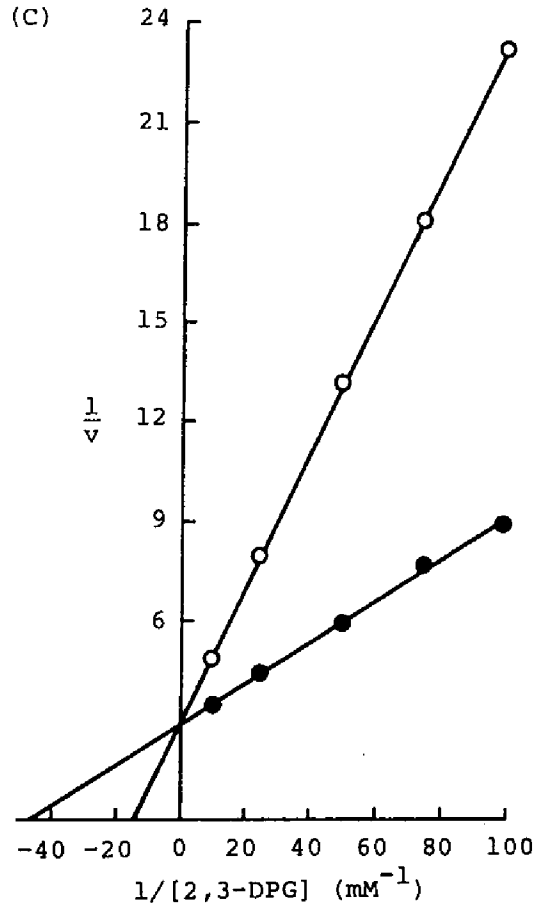


Fig. 21. Kinetics of the PGA mutase of peaks I, II and III.

(A) Peak I (0.5 µg); (B) peak II (6.5 µg); (C) peak III (36 µg). The concentration of DL-2-PGA was 4.95 mM (●—●) and 13.2 mM (O—O). Assay conditions are described in the text. v is expressed as the number of µmole 3-PGA produced per min.

Table 7. Apparent Km Values for the 2,3-DPG of the PGA mutase in Peaks I, II and III.

Apparent Km values for 2,3-DPG were calculated from intercepts on the abscissa in Fig. 21 at two concentrations of DL-2-PGA.

Enzyme used	Apparent Km at DL-2-PGA concn:	
	4.95 mM	13.2 mM
	μM	
Peak I	9.6	22.2
Peak II	9.4	27.8
Peak III	22.2	71.4

indicates that the PGA mutase of peak III has a larger Km value for 2,3-DPG or a smaller Ki value for 2-PGA than do peaks I and II, because the apparent Km value is expressed as $Km(1 + [I]/Ki)$ [57,68].

DISCUSSION

Until now, the purification of DPG mutase [14,38] and DPG ase [35-37] from human red cells have been independently attempted with little consideration for the possibility that the two activities are due to a common protein. Much attention has been paid to whether DPG ase is separable from PGA mutase or not [35-37], because the PGA mutases from various sources have DPG ase activity [49,62,70-75]. It has been revealed that there is DPG ase in human red cells which is separable from the phosphatase activity, probably due to the PGA mutase protein [35-37,76].

The purified DPG ase preparations, however, were not free from PGA mutase activity.

This chapter has described the simultaneous purification of DPG mutase, DPG ase and PGA mutase from human red cells. The most notable of these results is that the main DPG mutase and DPG ase activities (peak III) are displayed by the same enzyme protein. In addition, the enzyme molecule had PGA mutase activity. Our conclusion is based on the following observations. (a) The DPG mutase/DPG ase activity ratio was nearly constant throughout the purification steps (see Table 5). (b) Both activities on chromatography and isoelectric focusing behaved as if they were due to the same protein. (c) The final preparation displayed a single band on polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate and was homogeneous on ultracentrifugal analysis. (d) The DPG mutase, DPG ase and PGA mutase activities of peak III were lost at the same rate on thermal inactivation.

From the high yield of DPG mutase and DPG ase activities in each of the purification steps, the purified enzyme (peak III) seems to be responsible for the 2,3-DPG metabolism in human red cells. Furthermore, peak III has the known properties of both activities, *i.e.* activation or inhibition by various effectors (Table 6)[14,36,38,67].

Our results agree with a suggestion by Rosa *et al.* [61]. Rose has reported that DPG mutase is concentrated around pH 5.2 in isoelectric focusing [38]. Although this preparation had PGA mutase and DPG ase activities, the latter was not ordinarily

displayed in the absence of 2-phosphoglycolate [38]. Our final preparation obtained by isoelectric focusing of peak III had not only the DPG mutase and PGA mutase activities but also DPG ase activity which could be displayed in the absence of 2-phosphoglycolate (see Table 6).

As described in Chapter 5, it has become clear that "the multifunctional enzyme" which can display the three enzyme(DPG mutase, DPG ase and PGA mutase) activities exists not only in human erythrocytes but also in other cells such as pig erythrocytes, rabbit muscle and yeast.

It is unlikely that the PGA mutase activity of peak III plays an important role in glycolysis in human red cells, because other main PGA mutases(peaks I and II) with higher activities have been found. At present, the significance of the presence of chromatographically distinct PGA mutases(peaks I and II) is not clear. The separation of the two peaks may be due to a difference in net charge between the phosphorylated and dephosphorylated enzyme or indicate the presence of isozymes of PGA mutase in red cells.

So far as we have tested, no remarkable difference in enzymic properties between peaks I and II could be found. The higher DPG mutase or DPG ase activity ratio of peak II, as compared with that of peak I, could be caused by a small contamination of peak III in peak II. However, it is very likely that not only the PGA mutase protein of peak I but also that of peak II has intrinsic DPG mutase and DPG ase activities.

Thus, peaks III and I (probably also peak II) have three en-

zyme activities in different ratios. The structural analyses of these enzymes are in progress to get information on structural difference which may cause the appearance of the enzymes with differing ratios of three activities. It would be useful for understanding of genetic relationship of the enzymes.

CHAPTER 4 Multifunctional Enzyme-Phosphoglycerate Mutase/ 2,3-Diphosphoglycerate Phosphatase/Diphosphoglycerate Mutase from Human Erythrocytes Evidence for a Common Active Site

Simultaneous purification of DPG mutase, DPG ase and PGA mutase from human red cells has led to the conclusion that the three enzyme activities are displayed by the same enzyme protein (Chapter 3). Human red cells contain at least two kinds of the enzyme protein with different ratios of the three enzyme activities. Peak I is extremely high in PGA mutase, whereas peak III has high DPG mutase and DPG ase activities which correspond to about 95% of their total activities in red cells. Peak I probably functions as PGA mutase in the glycolysis and peak III is chiefly responsible for the 2,3-DPG metabolism.

For the better understanding of not only the regulatory mechanism of 2,3-DPG metabolism but also of the mechanism by which one enzyme catalyzes three kinds of reactions, it is necessary to reveal whether the three activities of peak III enzyme are exhibited at a common active site or different sites.

This chapter gives evidence that the three activities are attributable to a common active site at which one amino group essential for the binding of diphosphoglycerates is located.

MATERIALS AND METHODS

Peak III Enzyme

The procedures for purification of peak III enzyme from

human erythrocytes were as described in Chapter 3. The enzyme was stored as a precipitate in 75% ammonium sulfate. The enzyme protein was collected by centrifugation and was dissolved in 25 mM borate buffer pH 7.65 containing 0.1 mM EDTA. To remove ammonium sulfate, the enzyme solution was passed through a Sephadex G-50 column (1 x 27 cm) equilibrated with the above buffer. These procedures were carried out at 0-5°C. The enzyme solution was freshly prepared on the day of the experiment. A value of 61,000 (Chapter 3) was used as the molecular weight of the enzyme for calculations.

Other Materials

2,3-DPG, 3-PGA, DL-glyceraldehyde 3-phosphate and glyceraldehyde-3-phosphate dehydrogenase were obtained from Boehringer-Mannheim. NAD^+ and phosphoglycolate were from Sigma. DL-2-PGA was synthesized and purified by the methods described in Chapter 1. 1,3-DPG was synthesized enzymatically and then purified with a modification of Rose's method [14]. TNBS and sodium dodecyl sulfate were purchased from Wako Pure Chemical Industries, Ltd. DTNB was from Nakarai Chemicals, Ltd.

Enzyme Assays

DPG mutase (by Assay I), DPG ase (by Assay I) and PGA mutase were assayed as described in Chapter 3.

Inactivation by Trinitrobenzenesulfonate

Trinitrophenylation of the enzyme was performed in borate buffer containing KCl and NaCl. These salts were added to simulate intracellular conditions. Details of experimental con-

ditions are described in the legends of figures and tables in this chapter.

Quantification of Trinitrophenyl Group Incorporation

The extent of trinitrophenylation was monitored directly in the inactivation mixture at 367 nm, assuming a molar absorption coefficient of 10,800 $M^{-1}cm^{-1}$ for the trinitrophenyl group [77,78]. All spectrophotometric measurements were made in a Shimazu double-beam spectrophotometer UV-200 against an appropriate blank.

Sulfhydryl Determination

By using DTNB, the sulfhydryl groups were determined according to the method of Ernest [79,80]. At various times during trinitrophenylation, a 0.3 ml aliquot was diluted into 0.9 ml of 40 mM borate buffer pH 8.0 containing 2 mM EDTA and 5 mM lysine. Lysine instantly stopped further trinitrophenylation of the enzyme by consuming excess TNBS. After extensive dialysis against 40 mM borate buffer pH 8.0 containing 2 mM EDTA, the sample was adjusted to contain 2% sodium dodecyl sulfate and then an excess of DTNB was added. The absorbance at 412 nm was determined against a protein blank and a molar absorption coefficient of 13,600 $M^{-1}cm^{-1}$ was used to calculate the moles of sulfhydryl group present [81].

Determination of Molar Absorption Coefficient of Peak III Enzyme

A molar absorption coefficient of peak III enzyme at 280 nm was determined by measuring absorbance at 280 nm and the nitrogen content of the enzyme solutions. The nitrogen content was

determined by a micro-Kjeldahl method. Prior to digestion, absorbance of the enzyme solutions was measured at 280 nm. The protein concentrations of the enzyme solutions were calculated on the assumption that the nitrogen content of the peak III enzyme protein was 17%, an average value for many proteins. From the values of the absorbance of the enzyme solutions and those of the protein concentrations, a value for $A^{1\%}_{280\ nm}$ was calculated to be 16.0 $\pm$ 0.4. The molar absorption coefficient at 280 nm was calculated to be 9.8 $\pm$ 0.2 x 10^4 $M^{-1}cm^{-1}$ from the molecular weight, 61,000, of the enzyme (Chapter 3).

RESULTS

Inactivation of the Three Enzyme Activities by Trinitrophenylation

When peak III enzyme was incubated with TNBS, the DPG mutase, DPG ase and PGA mutase activities were lost at the same rate, as shown in Fig. 22. A plot of log residual activity(%) *versus* the incubation time is linear, indicating that the inactivations follow apparent first order kinetics. The presence of 2,3-DPG in the incubation mixture reduced the inactivation rates of the three enzyme activities to the same extent. It should be noted that 2,3-DPG is a substrate for both DPG ase and PGA mutase, and acts as an inhibitor which is competitive with 1,3-DPG in the DPG mutase reaction [14,39]. If it can be demonstrated that the trinitrophenylated amino group or groups are located at the active site, it will be evident that the three

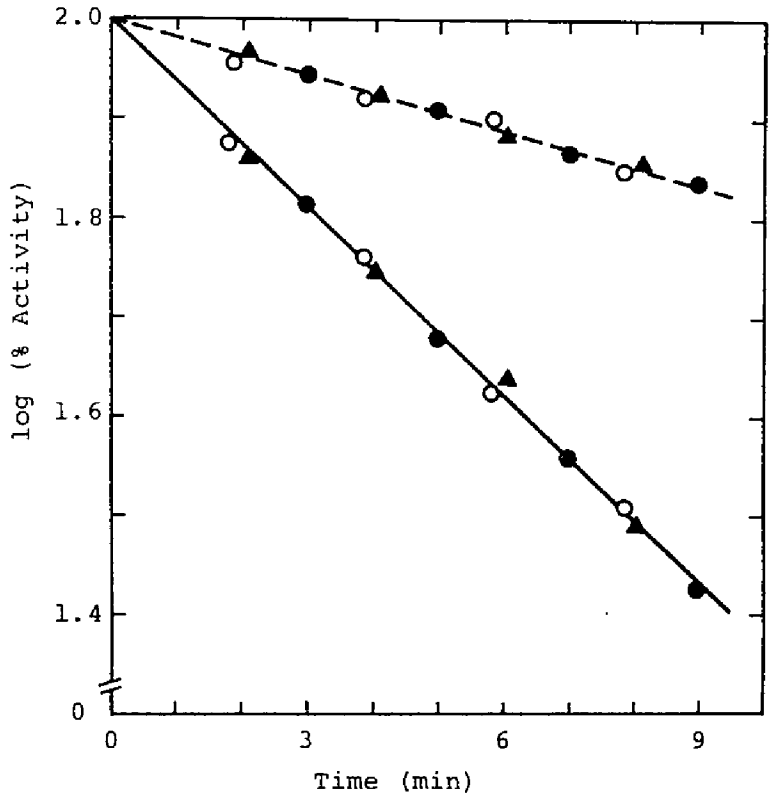


Fig. 22. TNBS-inactivation of the three enzyme activities.

The inactivation mixture contained 1 μM enzyme, 0.08 mM TNBS and 0.4 mM 2,3-DPG(-----) or no 2,3-DPG(——) in buffer A (25 mM borate buffer pH 7.65 containing 0.1 mM EDTA, 0.13 M KCl and 0.02 M NaCl). The temperature was 25°C. At appropriate times, aliqupts were removed for assays (0.2 ml for PGA mutase, 0.02 ml for DPG mutase and DPG ase). Per cent activity was calculated on the basis of activity obtained in the absence of TNBS. Enzyme activities were: DPG mutase(O), DPG ase(▲) and PGA mutase(●).

enzyme activities are exhibited at a common active site.

Extent of Trinitrophenylation and Loss of the Enzyme Activities

Fig. 23A shows time courses of both trinitrophenylation and of the loss of the PGA mutase activity of the enzyme. Rapid inactivation was observed in the absence of 2,3-DPG, while the

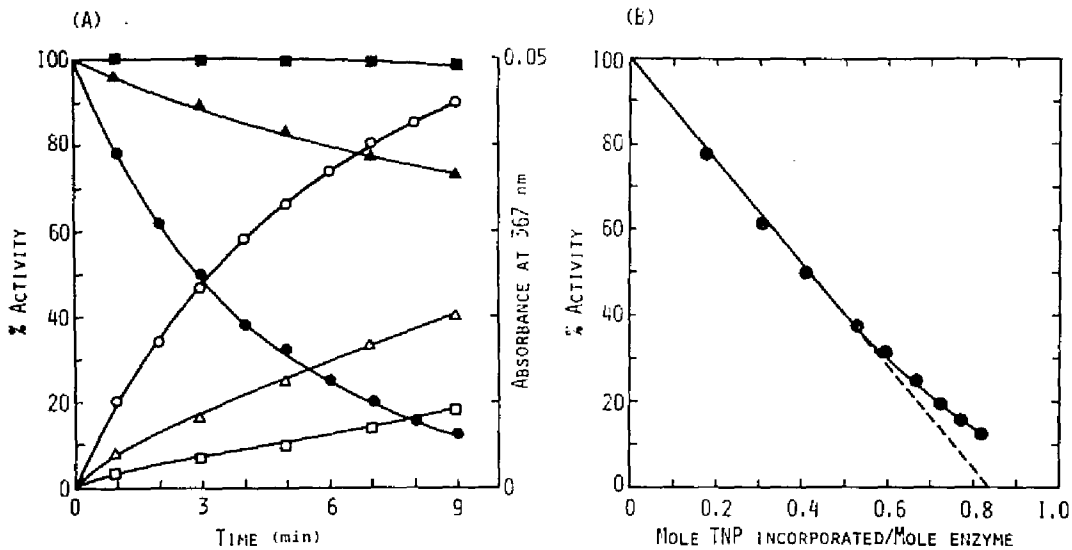


Fig. 23. (A) Time course of trinitrophenylation and loss of the PGA mutase activity of the enzyme.

The reaction mixture contained 5.1 μM enzyme, 0.12 mM TNBS and various concentrations of 2,3-DPG in buffer A (see legend for Fig. 22). The temperature was 25°C. The absorbance at 367 nm was monitored continuously against an appropriate protein blank to determine the extent of trinitrophenylation. At various times, a 0.05 ml aliquot was removed for the PGA mutase assay while absorbance at 367 nm was noted. Per cent activity was based on the activity obtained in the absence of TNBS. Enzyme activities were ● , ▲ and ■ . Absorbances at 367 nm were ○ , △ and □ . The concentrations of 2,3-DPG were: none (● , ○), 1 mM(▲ , △) and 5mM(■ , □).

(B) Residual activity *versus* mole of trinitrophenyl group incorporated per mole of enzyme in the absence of 2,3-DPG.

Values shown in (A)(● , ○) were replotted. Moles of the trinitrophenyl group incorporated per mole of enzyme were calculated as described in "Materials and Methods".

presence of 5 mM 2,3-DPG strongly repressed the trinitrophenylation and protected the enzyme almost completely from inactivation. In the absence of 2,3-DPG, inactivation and trinitrophenylation show a linear relationship up to 60% inactivation of the enzyme (Fig. 23B). Extrapolation of the linear portion of this curve to zero activity reveals that incorporation of about 0.82 mole

Table 8. Sulfhydryl Cotent of Peak III Enzyme during Trinitrophenylation.

The reaction mixture for trinitrophenylation was the same as in the experiment of Fig. 23. At various times during incubation, a 0.05 ml aliquot was removed for the PGA mutase assay and a 0.3 ml aliquot for the determination of sulfhydryl content. Sulfhydryl determination were carried out as described in "Materials and Methods". Per cent activity was based on the activity obtained in the absence of TNBS.

Incubation time (min)	Per cent activity	Sulfhydryl group/mole enzyme
0	100	5.7 ± 0.3
2.5	55	5.7
4.0	38	5.6
8.0	15	5.8

of trinitrophenyl group per mole of enzyme inactivates the enzyme completely. Deviation from unity probably originates in an error in calculating $A^{1\%}_{280\ nm}$ or in an erroneous molecular weight or both. Although TNBS is generally considered to be an amino-specific reagent, at high concentrations, it reacts with thiol groups [82]. In order to ascertain that the inactivation resulted in modification of an amino group, the sulfhydryl content was determined for the native and for the trinitrophenylated enzymes. The sulfhydryl content remained constant at the value of 5.7 for the native enzyme through 85% inactivation (Table 8). It is concluded from these results that the inactivation by TNBS is attributable to trinitrophenylation of one amino group which is essential for the three enzyme activities.

Effects of Phosphate Compounds on TNBS-Inactivation

Table 9 summarizes effects of phosphate compounds on inacti-

Table 9. Effects of Phosphate Compounds on TNBS-Inactivation and Their Known Functions in the Three Enzyme Reactions.

The reaction mixture contained 2.0 μM enzyme, 0.1 mM TNBS and the compounds at indicated concentrations in buffer A(see legend for Fig. 22). After incubation for 8 min at 25°C, a 0.2 ml aliquot was removed for the PGA mutase assay. Per cent activity was based on the activity obtained in the absence of TNBS. N; not known

Compound added	Concn (mM)	Per cent activity	Functions in the three enzyme reactions		
			DPG mutase	DPG ase	PGA mutase
None	—	24	—	—	—
2,3-DPG	0.27 1.00 5.00	53 80 97	Inhibitor [14, 39](Chapter 3)	Substrate	Substrate
1,3-DPG	0.02 0.08 0.23	40 47 57	Substrate	N	N
3-PGA	0.10 2.50 10.00	24 50 74	Substrate	Inhibitor [36] (Chapter 3)	Substrate
2-PGA	0.10 2.70 5.00	25 46 59	Substrate and Activator [14]	Inhibitor [36]	Substrate
Pi	6.25	25	Inhibitor [14]	Activator [36]	Inhibitor
2-Phospho-glycolate	1.25	30	Inhibitor [14] (Chapter 3)	Activator [36] (Chapter 3)	Inhibitor

vation of the PGA mutase activity by TNBS and their known functions in the three enzyme reactions. It was confirmed that the DPG mutase and DPG ase activities showed the same pattern as the PGA mutase activity regarding inactivation with TNBS in the presence and absence of the compounds tested. Of the three activities, the PGA mutase activity could be measured most accurately, since it was relatively insensitive to the phosphate compounds brought from the reaction mixtures for trinitrophenylation. For this reason, the PGA mutase activity is expressed as a representative of the three activities.

A significant protective effect was observed in the presence of 2,3-DPG or 1,3-DPG.

Monophosphoglycerates did not protect the enzyme from inactivation at the concentrations which would be adequate based upon Km value of DPG mutase [39] and Ki value of DPG ase [36]. At high concentrations, monophosphoglycerates exerted a protective action.

Inorganic phosphate and 2-phosphoglycolate were not protective. Both compounds were stimulatory toward DPG ase (Chapter 3, [36]) and inhibitory toward DPG mutase (Chapter 3, [14]) and PGA mutase.

Since enzymatic hydrolysis of 2,3-DPG is negligible under the conditions used for chemical modification, it is evident that the protective effect in the presence of 2,3-DPG results from the binding of this compound with the enzyme.

The 1,3-DPG preparation always contains 3-PGA and inorganic phosphate because of the lability of the acyl-phosphate group in

Table 10. Protective Effect of 1,3-DPG on TNBS-Inactivation.

The reaction mixture contained 0.25 μM enzyme, 0.7 mM TNBS, 0.1 mM EDTA, 0.13 M KCl, 0.02 M NaCl and 1,3-DPG or 2,3-DPG at the indicated concentrations in 25 mM borate buffer pH 8.0. After incubation for 3.75 min at 0°C, the PGA mutase activity was assayed by using a 0.2 ml aliquot. Per cent activity was based on the activity obtained in the absence of TNBS.

1,3-DPG	Activity	2,3-DPG	Activity
mM	%	mM	%
0	17	0	17
0.043	47	0.010	22
0.140	53	0.035	28
0.250	74	0.063	34

1,3-DPG. Therefore, the DPG mutase activity of the enzyme can result in 2,3-DPG from the 1,3-DPG preparation without addition of 3-PGA. Since most of the added 1,3-DPG was converted rapidly to 2,3-DPG under the conditions in Table 9, the protective effect in the presence of 1,3-DPG might be due to the 2,3-DPG produced. In order to examine the effect of 1,3-DPG on inactivation of the enzyme by TNBS, experimental conditions were selected so as to repress the conversion of 1,3-DPG to 2,3-DPG as much as possible. Repression was partially fulfilled by carrying out the modification at low temperature and alkaline pH in the presence of a high concentration of TNBS. TNBS reacts with amino groups at alkaline pH more rapidly than at an acidic one [83]. Results under these conditions are summarized in Table 10. After incubation of the enzyme with TNBS for 3.75 min, the enzyme was inactivated to 17% of the original activity. The inactivation was greatly reduced by increasing the 1,3-DPG con-

centration in the reaction mixture. It was found from separate experiments that 15-25% of the 1,3-DPG initially added was converted to 2,3-DPG during the incubation for 3.75 min in the absence of TNBS. When 2,3-DPG was added to the reaction mixture at the concentrations corresponding to 25% of 1,3-DPG, the protective effect was weaker than that of 1,3-DPG. From these results, it is clear that 1,3-DPG is also capable of protecting the enzyme from inactivation by TNBS.

These results suggest that one amino group essential to the three activities plays an important role in the binding of diphosphoglycerates to the enzyme.

Protection by 2,3-DPG

A dissociation constant of the binary complex of the enzyme and 2,3-DPG was determined by analyzing the protective effect of the phosphoglycerate. The value obtained was compared with the kinetic constants of 2,3-DPG in the enzyme reactions.

The TNBS-inactivation of the enzyme is an apparent first order reaction in the presence and absence of 2,3-DPG (see Fig. 22). Based on the assumption that the protective effect of 2,3-DPG is due to competition with TNBS, the inactivation of the enzyme in the presence of 2,3-DPG is expressed with the following equation.

$$\frac{d[EI]}{dt} = k([E]_0 - [EI] - [E\ 2,3\text{-DPG}]) \qquad \text{Eq. 1}$$

where k is an apparent first order rate constant and E is the enzyme. $[E]_0$ and [EI] represent the initial concentration of the

enzyme and the concentration of the trinitrophenylated-inactive enzyme. The enzyme species are related to the dissociation constant(Kd) of the enzyme-2,3-DPG complex by Equation 2.

$$\frac{([E]_0 - [EI] - [E\ 2,3\text{-DPG}])[2,3\text{-DPG}]}{[E\ 2,3\text{-DPG}]} = Kd \qquad \text{Eq. 2}$$

From these equations, the following equations are obtained.

$$\log \frac{[E]_0 - [EI]}{[E]_0} = -k't \qquad \text{Eq. 3}$$

$$k' = \frac{k}{2.303} \cdot \frac{Kd}{Kd + [2,3\text{-DPG}]} \qquad \text{Eq. 4}$$

In order to estimate k' values, inactivation of the enzyme by TNBS was followed while varying the concentration of 2,3-DPG from 0 to 1.0 mM. As shown in Fig. 24A the inactivation is a first order reaction as predicted by Equation 3. From the slopes of the straight lines, k' values were calculated to be 0.075, 0.036, 0.020 and 0.012 min^{-1} for 0, 0.2, 0.5 and 1.0 mM 2,3-DPG. A plot of 1/k' *versus* the concentration of 2,3-DPG was linear (Fig. 24B) and yielded a dissociation constant(Kd) of 180 µM. These experiments were carried out in the presence of 0.13 M KCl and 0.02 M NaCl in order to simulate intracellular conditions of ionic strength and ion species involved [84]. The same kind of experiments were performed in the presence of 0.1 M KCl, because basic properties of DPG mutase and DPG ase have been studied at this ionic strength [36, 39]. The pattern of enzyme inactivation was similar to that of Fig. 24 (data not

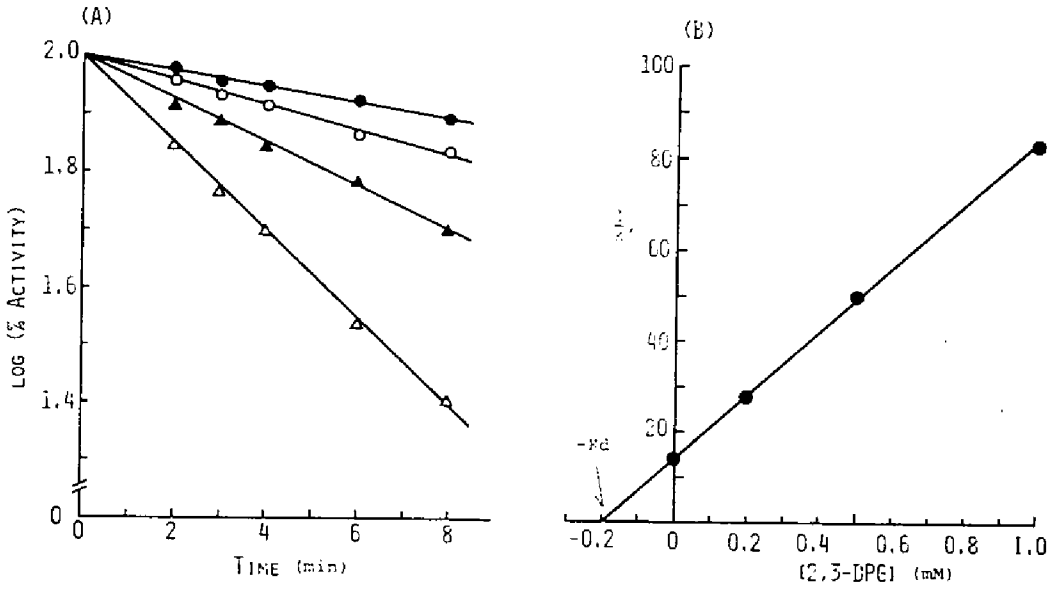


Fig. 24. Determination of the dissociation constant(Kd) of the 2,3-DPG-enzyme complex from the protective effect.

(A) The incubation mixture contained 7.0 μM enzyme, 0.1 mM TNBS and 2,3-DPG at the indicated concentrations in buffer A (see legend for Fig. 22). The temperature was 25°C. At various times, a 0.05 ml aliquot was removed for PGA mutase assay. Per cent activity was based on the activity obtained in the absence of TNBS. The concentrations of 2,3-DPG were: none(Δ), 0.2 mM (▲), 0.5 mM(O) and 1.0 mM(●). (B) A plot of 1/k' *versus* the concentration of 2,3-DPG. Values of k' were calculated from the slopes of lines in (A).

shown). Values of k' were 0.085, 0.050, 0.038 and 0.026 min^{-1} for 0, 0.05, 0.10 and 0.20 mM 2,3-DPG. A value for the dissociation constant(Kd) was calculated to be 82 μM.

Kinetic studies on the PGA mutase activity of peak III enzyme were performed in order to determine a Km value for 2,3-DPG. As shown in Fig. 25A, the PGA mutase was inhibited by substrate (2-PGA) competitively with respect to 2,3-DPG. The initial velocity(v) under these conditions is expressed by the following equation.

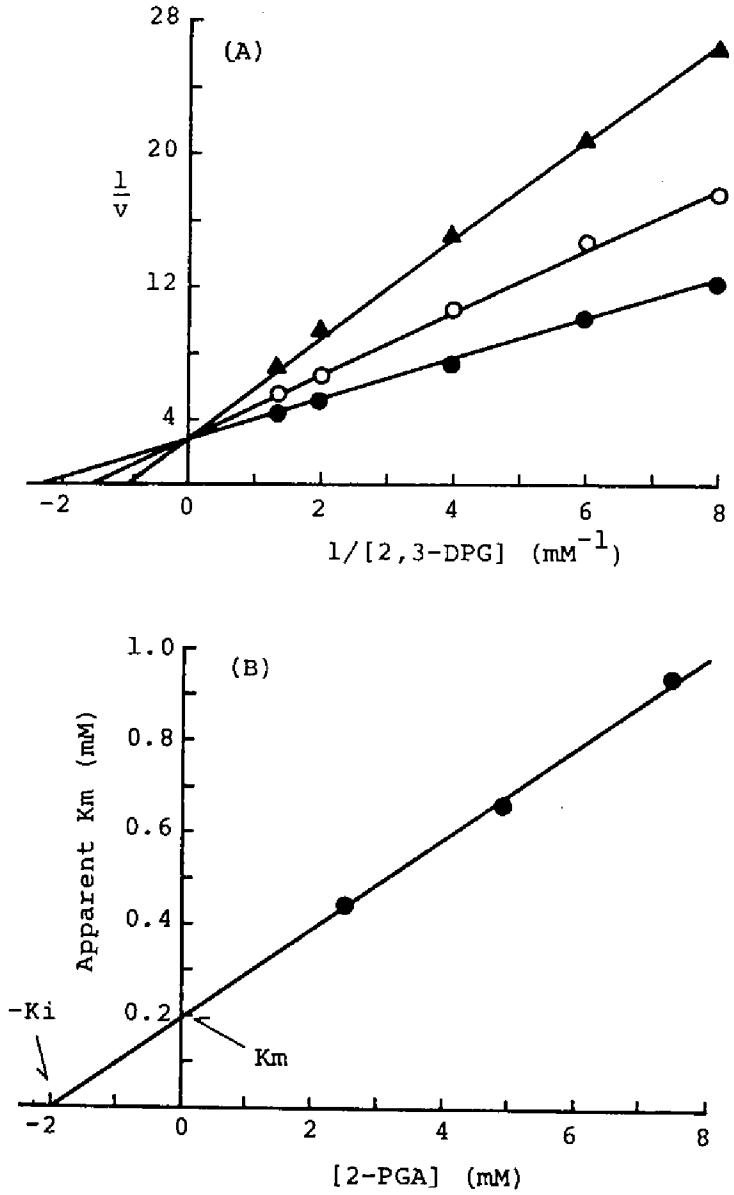


Fig. 25. Determination of Km value of 2,3-DPG from the kinetics of PGA mutase reactions.

(A) The reaction mixture contained 7.3 μM enzyme, 2-PGA and 2,3-DPG at indicated concentrations in 2.0 ml of buffer A(see legend for Fig. 22). The temperature was 25°C. The concentrations of 2-PGA were: 2.5 mM(●), 4.9 mM(O) and 7.5 mM (▲), while v is expressed as μmole 3-PGA produced per min. (B) A plot of the apparent Km value *versus* the concentration of 2-PGA. Apparent Km values were calculated from the intercepts on the ordinate in (A).

$$v = \frac{V}{\frac{Km}{[2,3\text{-DPG}]}\left(1 + \frac{[2\text{-PGA}]}{Ki}\right) + 1} \qquad \text{Eq. 5}$$

V and Ki are the maximum velocity and the inhibition constant of 2-PGA. Therefore, the apparent Km values for 2,3-DPG, obtained from the intercepts on the abscissa of Fig. 25A, are equal to Km(1 + [2-PGA]/Ki). Plotting the apparent Km values *versus* the concentration of 2-PGA gave a straight line (Fig. 25B). From the intercept on the ordinate, the Km value for 2,3-DPG was calculated to be 200 μM in the presence of 0.13 M KCl and 0.02 M NaCl. In the presence of 0.1 M KCl, the Km value decreased to 80 μM (data not shown).

These constants are summarized in Table 11. Good agreement of the kinetic constants with the dissociation constants leads to the conclusion that 2,3-DPG protects the enzyme through competition with TNBS for the one amino group located at the 2,3-DPG binding site.

Protection by Monophosphoglycerates

Kinetic studies on the PGA mutase activity in the previous section showed that 2-PGA could interact with the 2,3-DPG binding site. The protective effect of monophosphoglycerates at high concentrations may be attributable to the binding of the monophosphoglycerates to the diphosphoglycerate-binding site. Quantitative analyses were performed by comparing the inhibition constant of monophosphoglycerate in the PGA mutase reaction with the dissociation constant of the enzyme-monophosphoglycerate complex. The dissociation constant(Kd) was determined from the

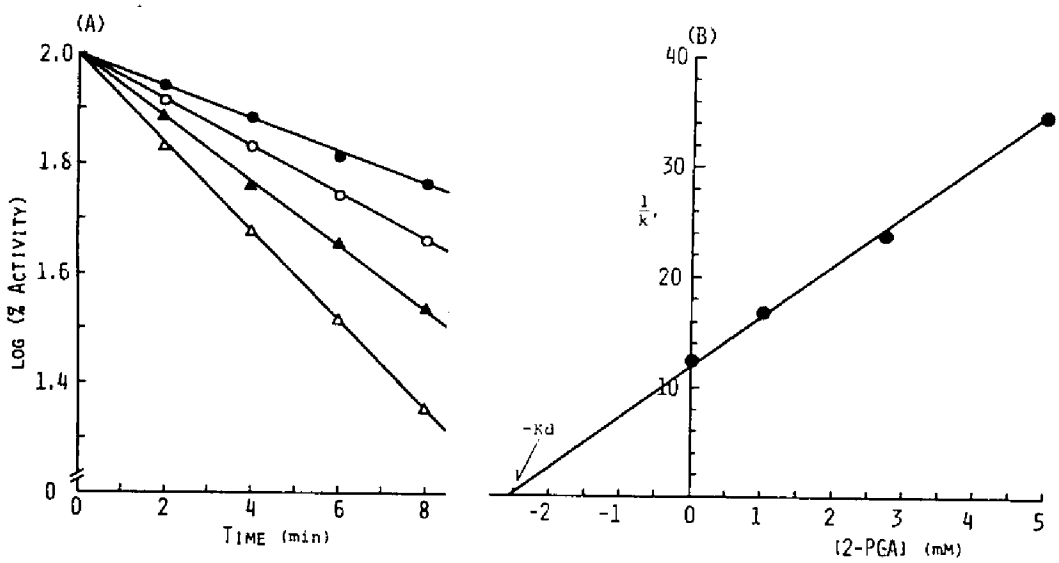


Fig. 26. Determination of the dissociation constant(Kd) of the 2-PGA-enzyme complex from the protective effect.

(A) The incubation mixture contained 2.0 μM enzyme, 0.1 mM TNBS and 2-PGA at indicated concentrations in buffer A(see legend for Fig. 22). The temperature was 25°C. At various times, a 0.2 ml aliquot was removed for the PGA mutase assay. Per cent activity was based on the activity obtained in the absence of TNBS. The concentrations of 2-PGA were: none(Δ), 1.1 mM(▲), 2.7 mM(O) and 5.0 mM(●). (B) A plot of 1/k' *versus* the concentration of 2-PGA. Values of k' were calculated from slopes of lines in (A).

protective effect of monophosphoglycerate in the same way as for 2,3-DPG. As shown in Fig. 26A, inactivation of the enzyme by TNBS in the presence of 2-PGA was an apparent first order reaction. From the slopes of the straight lines, k' values were calculated to be 0.080, 0.058, 0.042 and 0.029 min^{-1} for 0, 1.1, 2.7 and 5.0 mM of 2-PGA. A plot of 1/k' *versus* the concentration of 2-PGA was linear (Fig. 26B), and the dissociation constant(Kd) was determined to be 2.5 mM. The inhibition constant of 2-PGA(Ki) was calculated to be 2.0 mM from the intercept on the abscissa of Fig. 25B. Good agreement of the inhibition constant with the disso-

Table 11. Summary of Constants for 2,3-DPG and 2-PGA.

Kd is the dissociation constant obtained from chemical modification experiments. Km and Ki are kinetic constants obtained from PGA mutase reactions.

Compound	Constant	Condition	
		0.13 M KCl 0.02 M NaCl	0.10 M KCl
		μM	
2,3-DPG	Kd	180	82
	Km	200	80
		mM	
2-PGA	Kd	2.5	—
	Ki	2.0	—

ciation constant indicates that the protective effect of 2-PGA is through the direct interaction of this compound with the diphosphoglycerate-binding site (Table 11).

With 3-PGA, the dissociation constant was 2.6 mM (data not shown). Although the inhibition constant of 3-PGA was not determined, the dissociation constant was in the range in which the protective effect was observed (see Table 9).

DISCUSSION

Peak III enzyme from human red cells is a multifunctional enzyme which includes DPG mutase, DPG ase and PGA mutase, and which is responsible for the 2,3-DPG metabolism (Chapter 3). Trinitrophenylation of about one amino group per mole of enzyme resulted in complete loss of the three enzyme activities.

Diphosphoglycerates(2,3-DPG and 1,3-DPG) inhibited trinitrophenylation and protected the enzyme from inactivation. Quantitative analyses of their protective effects indicated that the amino group was a residue located at the diphosphoglycerate-binding site instrumental to the three enzyme activities.

Peak III enzyme is composed of two subunits of molecular weight 29,000 daltons (Chapter 3). Rose has shown with the DPG mutase from human erythrocytes that one phosphoryl group per subunit is covalently bound upon incubation of the enzyme with either 1,3-DPG or 2,3-DPG [38]. It is of interest that complete inactivation of the enzyme is achieved by trinitrophenylation of one amino group per dimer. These observations might be explained by the model of Levitzki *et al.* [85] in which binding of a ligand on one subunit hinders subsequent ligand binding of the neighbouring subunit. This type of interaction has been termed "half-of-the-sites reactivity" and is reported for several enzymes having subunit structure [85]. A conformational change of one subunit induced by the binding of TNBS may be transmitted to the neighbouring subunit so that neither TNBS nor diphosphoglycerates can bind to it.

Although further studies on the three enzyme activities are required to show clearly the mechanism by which they are exhibited at a common active site, a tentative model is presented based upon the results in this chapter and upon kinetic studies on DPG ase [36] and DPG mutase [14,38,39]. Peak III enzyme seems to possess two separable sites on the enzyme molecule. One is exclusively available for monophosphoglycerates and

another for diphosphoglycerates. Monophosphoglycerates can bind to the diphosphoglycerate site with the lower affinity. When the two sites are occupied by 3-PGA and 1,3-DPG, the DPG mutase reaction proceeds. With DPG mutase from human red cells, an ordered sequential pathway has been presented in which 1,3-DPG binds with the free enzyme, followed by the combination with 3-PGA to form a ternary complex [14]. 2,3-DPG is a potent inhibitor competitive with 1,3-DPG [14, 39]. If the two sites are occupied by 2 or 3-PGA and 2,3-DPG, the enzyme becomes PGA mutase. When the diphosphoglycerate site is filled by 2,3-DPG, the enzyme is converted into DPG ase. It has been shown that the filling the monophosphoglycerate site with 2,3-DPG is essential for the functioning of the DPG ase of yeast PGA mutase [49]. It is not known whether the monophosphoglycerate site is filled by 2,3-DPG when peak III protein functions as DPG ase. The inhibitory effect of monophosphoglycerates on DPG ase is due to the conversion of the enzyme into PGA mutase. Inorganic phosphate and 2-phosphoglycolate probably interact with the monophosphoglycerate site to stimulate DPG ase. These compounds are inhibitor for the other two enzyme activities. With this model, known functions of phosphate compounds in Table 9 seem to be interpretable to a great extent, although not completely.

It is unlikely that the PGA mutase activity of peak III enzyme plays an important role in glycolysis in human red cells, because peak I enzyme with a higher activity has been found (Chapter 3). Physiologically it is of importance that the two enzyme activities responsible for 2,3-DPG metabolism are dis-

played at a common active site on one protein. The kind of reaction which occurs depends on the affinities of substrates with the enzyme and on substrate concentrations in the cell. The diphosphoglycerate bound to the diphosphoglycerate site definitely selects the enzyme activity which will operate. In addition, as seen in the case of inorganic phosphate, the effect of a ligand on one of the two enzyme activities can be magnified by exerting the opposite effect on another one. Normally an irreversible reaction accompanying the consumption of a high energy bond and its reverse reaction are catalyzed by different enzymes. Regulation of the reactions is effectively achieved by an effector exerting one effect on one enzyme and the opposite effect on the other. Interconversion between fructose 1,6-diphosphate and fructose 6-phosphate is a case in which AMP activates phosphofructokinase and inhibits fructose-1,6-diphosphatase [86]. From this point of view, the regulatory mechanism proposed for 2,3-DPG metabolism in human red cells would be a new type of metabolic regulation achieved with one multifunctional enzyme capable of catalyzing two irreversible reactions.

CHAPTER 5 MULTIFUNCTIONAL ENZYME-PHOSPHOGLYCERATE MUTASE/ 2,3-DIPHOSPHOGLYCERATE PHOSPHATASE/DIPHOSPHOGLYCERATE MUTASE FROM VARIOUS SOURCES

It has been proved that 2,3-DPG-dependent PGA mutases from various sources have the DPG ase activity [49,62,70-75]. Human red cell PGA mutases(peaks I, II and III) have not only the DPG ase activity but also the DPG mutase activity and this multifunctional property of peak III enzyme is very important for the regulation of 2,3-DPG metabolism (Chapters 3 and 4).

In view of these points, the present investigation explores the possibility that a 2,3-DPG-dependent PGA mutase generally can catalyze the decomposition and the synthesis of 2,3-DPG.

MATERIALS AND METHODS

Chemicals and Enzymes

2,3-DPG, 3-PGA, DL-glyceraldehyde 3-phosphate, glyceraldehyde-3-phosphate dehydrogenase and rabbit muscle PGA mutase were obtained from Boehringer-Mannheim. NAD^+ was from Sigma. DL-2-PGA was synthesized and purified as described in Chapter 1. Yeast PGA mutase was purified and crystallized by a new procedure [87].

Enzyme Assays

PGA mutase, DPG ase (by Assay I) and DPG mutase (by Assay I) were assayed as described in Chapter 3.

One unit of enzyme activity is defined as the amount of enzyme which catalyzes the conversion of 1 μmole of the substrate to the product per min. Specific activity is expressed as units per mg of protein.

Protein Concentration

Protein was determined by measuring the absorbance at 280 nm. Its concentration was calculated from the absorbance at 280 nm based on the values of $A^{1\%}_{280\ nm}$ (rabbit muscle PGA mutase : 10.5, yeast PGA mutase : 14.2, pig erythrocyte protein : 10).

RESULTS

DPG mutase Activity of PGA mutases from Rabbit Muscle and Yeast

The third activity, DPG mutase activity, was searched in the PGA mutases from rabbit muscle and yeast. As shown in Table 12, both PGA mutase proteins were found to have not only PGA mutase and DPG ase activities but also DPG mutase activity. Their DPG ase and DPG mutase activities, however, were very low compared with PGA mutase activity. This multifunctional property observed in these enzyme proteins is same as that of peak I enzyme from human erythrocytes(Chapter 3).

Multifunctional Enzyme-PGA mutase/DPG ase/DPG mutase in Pig Erythrocyte

PGA mutase, DPG ase and DPG mutase were co-purified from pig erythrocytes, which containe high level of 2,3-DPG as well as human erythrocyte, in order to prove the existence of the

multifunctional enzyme-PGA mutase/DPG ase/DPG mutase. All steps were carried out at 0-4°C.

Hemolysate. To remove the serum, 3,500 ml of pig blood obtained from a butchery was centrifuged at 4,500 x g for 5 min. The red cells were washed three times with an equal volume of 0.9% NaCl containing 0.1 mM EDTA. The buffy coat was removed with suction. The packed cells(935 ml) were hemolysed by adding an equal volume of 10 mM Tris-HCl buffer pH 7.5 containing 0.2 mM EDTA and 10 mM 2-mercaptoethanol followed by freezing and thawing.

Bulk Separation on DEAE-Cellulose. To the hemolysates(1,870 ml), 130 g of DEAE-cellulose previously equilibrated with 5 mM Tris-HCl buffer pH 7.5 containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol(buffer A) was added and the mixture was stirred for 3 h. The cellulose with absorbed enzymes was filtered on a Buchner funnel and washed with successive, 4,000 ml of buffer A and 2,000 ml of buffer A containing 0.05 M KCl. Then, the enzyme was eluted by washing the cellulose with 2,000 ml of buffer A containing 0.35 M KCl. The eluate was brought to 75% saturation with solid ammonium sulfate.

Ammonium Sulfate Fractionation. The precipitate was collected by centrifugation at 6,900 x g for 20 min and was dissolved in 750 ml of buffer A. From the difference between the final volume(840 ml) of the solution and the amount of the buffer used, an estimate was made of the quantity of ammonium sulfate in the precipitate. To this solution, 124 g of solid ammonium sulfate was added to bring the salt concentration to 27.5% saturation.

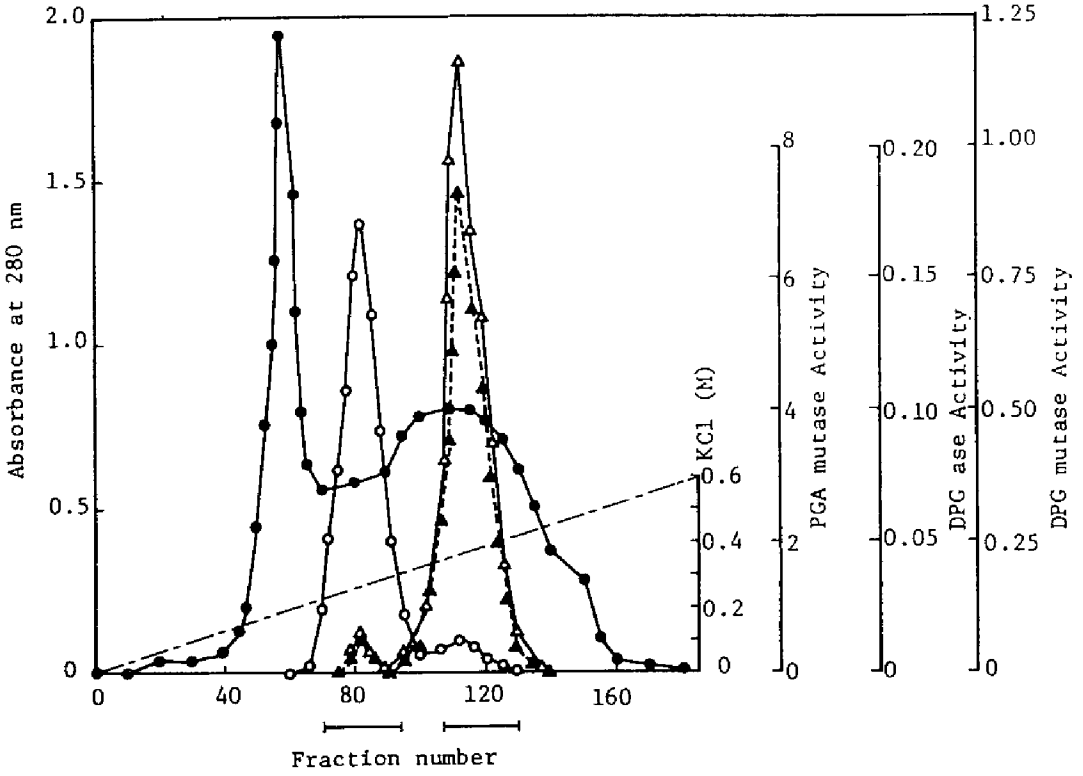


Fig. 27. Elution profile from DEAE-Sephadex.

(●—●) Absorbance at 280 nm; (△—△) DPG mutase; (▲--▲) DPG ase; (O—O) PGA mutase; (—·—·—) KCl concentration. Activities are expressed as units/ml. 1.56 g of the protein purified by ammonium sulfate fractionation was applied. Fractions were 20 ml. The flow rate was 3 ml/min. The horizontal lines indicate the fractions pooled after elution.

The precipitate was removed by centrifugation, and the supernatant was brought to 65% saturation by the addition of 251 g of solid ammonium sulfate.

DEAE-Sephadex Chromatography. The collected precipitate was dissolved in and dialysed against buffer A for 24 h. The dialysed solution was added to a column (4.8 x 33 cm) containing DEAE-Sephadex A-50 previously equilibrated with buffer A. The column was developed with a 4,000 ml linear gradient of KCl

Table 12. Multifunctional Enzyme-PGA mutase/DPG ase/DPG mutase from Various Sources.

Enzyme	Specific activity		
	PGA mutase	DPG ase	DPG mutase
Rabbit muscle PGA mutase	664	0.13	0.07
Yeast PGA mutase	1,000	0.17	0.04
Human erythrocyte peak I enzyme	763	0.28	1.01
peak III enzyme	10	2.01	9.78
Pig erythrocyte 1st peak	5.74	0.002	0.012
2nd peak	0.63	0.190	1.3

rainging from 0 to 0.65 M in buffer A. Fig. 27 represents a elution profile showing protein concentration and PGA mutase, DPG ase and DPG mutase activities. The three enzyme activities were separated into two peaks. The first peak(fractions 70-94) contained about 82% PGA mutase activity finally recovered and small amounts of DPG ase and DPG mutase activities(3-4%, respectively). Most of DPG ase and DPG mutase activities(85-88%) appeared in the second peak(fractions 107-130) which had also about 6% PGA mutase. Although the enzymes are not yet completely purified, these results strongly suggest that pig erythrocyte as well as human erythrocyte (Chapter 3) has two types of the multifunctional enzyme-PGA mutase/DPG ase/DPG mutase: one is high PGA mutase type and another one is high DPG ase and DPG mutase type. The three enzyme activities of the two peaks are shown in Table 12.

DISCUSSION

The data summarized in Table 12 led to the conclusion that the 2,3-DPG-dependent PGA mutase generally exists as a multifunctional enzyme-PGA mutase/DPG ase/DPG mutase. Ratios of the three enzyme activities are variable depending on enzyme sources. As shown in Table 13, the author proposes to classify the multifunctional enzymes into two groups, Class I and Class II: Class I enzyme has high PGA mutase activity and Class II enzyme has high DPG ase and DPG mutase activities. Therefor, PGA mutases from rabbit muscle and yeast, peak I enzyme from human erythrocytes(Chapter 3) and the first peak from pig erythrocytes belong to Class I, while peak III enzyme from human erythrocytes(Chapter 3) and the second peak from pig erythrocytes belong to Class II. In human and pig erythrocytes, Class I

Table 13. Classification of the Multifunctional Enzyme-PGA mutase/DPG ase/DPG mutase.

Class	Activity		
	PGA mutase	DPG ase	DPG mutase
Class I enzyme	High	Low	Low
	Rabbit muscle PGA mutase, Yeast PGA mutase, Human erythrocyte peak I enzyme, Pig erythrocyte first peak		
Class II enzyme	Low	High	High
	Human erythrocyte peak III enzyme, Pig erythrocyte second peak		

enzyme probably functions in glycolysis and Class II enzyme, in the 2,3-DPG metabolism which is maintaining 2,3-DPG at a high level. However, it is not established whether Class I enzymes from rabbit muscle and yeast are responsible not only for glycolysis but also for 2,3-DPG metabolism in those cells containing 2,3-DPG at low levels.

A common feature, one protein displays the three enzyme activities, makes it possible to assume that Class I and Class II enzymes are coded by one gene, *i.e.* the proteins originally have identical structures. Based on this assumption, the author is considering the mechanism of isomerization of the enzyme as follows. The structure of Class I enzyme would correspond to the original structure. After the gene expression, the cells which require high 2,3-DPG level(*e.g.* human and pig erythrocytes) convert some of Class I enzymes into Class II enzymes in order to maintain 2,3-DPG at high levels. To obtain more informations on these problems, attempts to bring about the interconversion between Class I and Class II enzymes and structural analyses of these enzymes are in progress.

Summary

Chapter 1

A procedure for microdetermination of 2,3-DPG, utilizing its role as coenzyme in the PGA mutase reaction is described. The coenzymic activity was determined by assaying PGA mutase polarimetrically without a coupled enzyme. This method is applicable to samples containing as little as 0.002 μmole of 2,3-DPG/ml. The content in various biological extracts was determined.

Chapter 2

A direct method for simultaneously estimating the activity of PGA mutase and the concentration of 2,3-DPG in tissue homogenates and in the hemolysates of mammals was established. By this method, a marked increase in the activity of PGA mutase was observed in the red cells of patients with anemia and congestive heart failure, while the concentration of 2,3-DPG was in the normal range.

Chapter 3

DPG mutase, DPG ase and PGA mutase have been purified from human red cells. Three enzymes were co-purified throughout all purification steps. Three fractions(peaks I, II and III) which were chromatographically separable and had three activities in different ratios were obtained.

Peak III which contained the main DPG mutase and DPG ase activities was purified to homogeneity by electrophoretic and

ultracentrifugal analyses. The homogeneous preparation had the PGA mutase activity. The three activities were lost at the same rate during thermal inactivation. Thus, DPG mutase and DPG ase activities, which are responsible for 2,3-DPG metabolism in red cells, are displayed by the same enzyme protein which has PGA mutase activity.

Peaks I and II were rich in the PGA mutase activity. Both peaks showed DPG mutase and DPG ase activities, although these two activities were much smaller than those of peak III.

Some of the enzymic properties of peak III are described. Comparative studies on three peaks showed that the PGA mutase of peak III differed from that of peaks I and II in the kinetic property and thermostability.

Chapter 4

Multifunctional enzyme-PGA mutase/DPG ase/DPG mutase which is responsible for 2,3-DPG metabolism in human red cells (peak III enzyme) was subjected to chemical modification by TNBS. The three enzyme activities were inactivated by TNBS at the same rate. The sulfhydryl content of the enzyme was unchanged during trinitrophenylation, indicating that derivatization was through the amino group. Trinitrophenylation of about one amino group per mole of the enzyme resulted in complete loss of the three activities.

Both 2,3-DPG and 1,3-DPG inhibited trinitrophenylation and effectively protected the enzyme from inactivation. Although monophosphoglycerates did not show any protective effect at con-

centrations which should be adequate based upon their kinetic constants, they were protective at higher concentrations.

Inactivation by trinitrophenylation was an apparent first order reaction. The dissociation constant of the enzyme-2,3-DPG complex was determined by analyzing the first order reaction on the assumption that the protective effect of 2,3-DPG was due to a competition with TNBS. The dissociation constant was in good agreement with kinetic constants of 2,3-DPG in the enzyme reactions, which indicated that 2,3-DPG did indeed exert its protective effect through competition with TNBS for an amino group of the enzyme. The protective effect of monophosphoglycerates could be rationalized with kinetic evidence that 2-PGA at high concentrations interacts with the 2,3-DPG binding site.

These results indicate that the enzyme exhibits the three enzyme activities at a common active site at which one amino group essential for binding of diphosphoglycerates is located. Based on the multifunctional properties of this enzyme, a possible mechanism was discussed for regulation of 2,3-DPG metabolism in human red cells.

Chapter 5

PGA mutases from rabbit muscle and yeast have been found to have not only PGA mutase and DPG ase activities but also DPG mutase activity.

PGA mutase, DPG ase and DPG mutase were partially co-purified from pig erythrocytes. Elution profiles of the three enzyme activities on DEAE-Sephadex column chromatography strongly

suggested that pig erythrocyte as well as human erythrocyte has two types of the multifunctional enzyme-PGA mutase/DPG ase/DPG mutase: one is high PGA mutase type and another one is high DPG ase and DPG mutase type.

These results led to the conclusion that a 2,3-DPG-dependent PGA mutase generally exists as a multifunctional enzyme-PGA mutase/DPG ase/DPG mutase. The author classified these multi-functional enzymes into two groups; Class I enzyme(high PGA mutase type) and Class II enzyme(high DPG ase and DPG mutase type).

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